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FLP recombinase-steroid receptor fusion proteins convert ligand binding into DNA recombination in yeast and mammalian cells. We describe a ligand responsive FLP-estrogen receptor binding domain (FLP-EBD) in yeast to follow ligand binding interactions. Shortening the distance between FLP and the EBD in the fusion protein specifically blocks induction only by antagonists, presenting an assay for them in yeast. We show using this assay for ligand binding that correlates with transcriptional responsiveness, that agonists and antagonists differently position the C-terminus of the ligand binding domain (helix 12) and the F domain. Upon antagonist binding, the F domain interferes with the fusion protein activity. Mutational disruption of helix 12 alters the position of the F domain, imposing interference after agonist or antagonist binding. Numerous genetically selected inversion mutations where now agonists, but not antagonists, induce interference are similarly reliant on helix 12 and F domain positioning. Our results demonstrate that agonists and antagonists differently position helix 12 and implicate the F domain in mechanisms of antagonist action. Mutagenesis of the EBD has generated numerous mutations with altered ligand specificity. This study is consistent with the conclusions of the recent x-ray structures of a static EBD to define specific amino acid contacts in the ligand binding pocket, namely at E353, R394, G521, H524, & L540. The structures clarify and aid new drug design.

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Mark D. Nichols Sept. 5, 1998

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TABLE OF CONTENTS

A Genetic Screen for Ligand Binding by the Human Estrogen Receptor.

Introduction

Estrogens regulate target cell proliferation and gene transcription through a pathway initiated by binding to the estrogen receptor (ER), a member of the steroid hormone receptor class of nuclear receptors (3,14). A diverse group of antagonistic ligands have been identified which interfere with ER activation (12,26,46). Prominent among these are the clinically relevant antagonists, 4-hydroxytamoxifen (Z-OHT), raloxifene and ICI 182,780. Biochemical and functional work on the mechanisms of antagonist action has led to the conclusion that they induce different conformations of the ER ligand binding domain (domain E) than agonists (1). They also exhibit a spectrum of activities, presumably a reflection of the multiple steps involved in the ER activation pathway and the diversity of responses (see 26,28). Molecular details of the differences between agonist and antagonist activities are now becoming clear.

Steroid receptors are modular proteins possessing two highly conserved domains, termed the C and E domains (3,15). They are joined by an unstructured and varied D domain sequence (15). Domain C is 66 amino acids long with two zinc fingers, which mediate sequence-specific DNA binding. Domain E, also known as the ligand binding domain (LBD), is about 240 amino acids long (50) and mediates numerous overlapping functions: ligand binding, dimerization, Hsp90 binding, transrepression, transcriptional activation and cellular localization (3,25). Mutations within LBDs have revealed some insight into the structure/function relationship, and x-ray crystal structures, especially the recent ones of the estrogen receptor LBD present a framework for understanding how LBDs function (6,7,35,38,45,49). The E domain can be fused onto other proteins to impose ligand dependency on their activity (36). Here we make use of our observation that steroid regulation can be imposed on the enzyme activity of a site-specific recombinase, FLP, by expressing FLP/steroid receptor fusion proteins (FLP-LBDs; 33). FLP-LBDs are inactive as recombinases in the absence of a cognate ligand and respond to both agonists and antagonists in a concentration dependent manner (33), resulting in a fixed change in reporter gene DNA which converts ligand

binding to an enzyme activity. Thus FLP-LBDs faithfully reflect the initially repressed, unliganded, state of steroid receptors but do not discriminate between agonists and antagonists. Hence the differences between these two classes of ligands must occur after ligand-induced release from the initially repressed state (33). For example, two domains of the estrogen receptor protein are capable of transcriptional activation functions (AFs): a hormone-independent AF-1 in the A/B domain and the hormone-dependent AF-2 at helix 12 within the E-(LBD) domain (21). AF-1 and AF-2 functions vary in importance with individual promoters and cell-types and the primary determinant of AF-2 activity depends on whether the bound ligand is an agonist or an antagonist (4,26,27,47). Consequently, FLP-LBDs present assays where ligand activities are measured directly, by site-specific recombination, and the multiple steps and specificities involved in ligand-mediated transcriptional responses are circumvented. Here, a FLP-estrogen binding domain fusion protein assay in yeast, where no AF-2 interacting transcription cofactors have been found (4), is used to address conformational differences induced by agonists and antagonists.

The amount of estrogen receptor D domain included in the fusion protein between the FLP and EBD domains influences the activity of the recombinase. If the D domain is omitted, antihormones but not hormones, are unable to activate the fusion recombinase (33). The ability to distinguish between hormones and antihormones in yeast is extremely useful in understanding how ligands activate the estrogen receptor. The objective of this effort has been to exploit yeast-based genetic screens of mutated EBD clone banks to find mutations which specifically alter ligand binding, and hence recombination. By use of different ligands and various concentrations, we find specific amino acid changes that alter ligand induced function, thereby defining components of binding. Combining the EBD fusion point dependence for antihormone action with numerous random mutations of the estrogen binding domain has produced EBDs which show altered or reversed activation by hormones and antihormones. The results are consistent with and add biological data to confirm conclusions drawn from the recent x-ray structures of the ER LBD with estradiol or raloxifene (7). Our strategy separates multiple functions present in the estrogen binding domain and should help the potential for rational drug design.

Objectives

This research involves defining the interactions between estrogen ligands and the estrogen binding domain to ascertain a better understanding of ligand binding and function, especially as that relates to hormone and antihormone function. The fusion point dependence for antihormone action in our FLP recombinase/ estrogen binding domain (FLP-EBD) system in yeast allows a mechanism to study therapeutically important antihormones, such as tamoxifen and raloxifene, and why/how they act as antihormones. We find that agonists and antagonists position the C-terminal part of the ligand binding domain (helix 12) and the F domain differently, leading to their known opposite effects on transcription by ER. Helix 12 is the most important region for AF-2 function and cofactor binding for transcription. Numerous mutations in the EBD also alter a subset of ligand interactions. Many of these important effects coincide nicely with the recently published structure of the ER LBD in the presence of both a hormone and an antihormone (7). The ligand binding pocket and molecular contacts are now well defined and should allow better drug design for improved pharmaceutical agents for ER.

BODY

This report covers the grant, DAMD17-94-J-4103 (Sept 94-Aug 98). The work has involved characterizing FLP recombinase-estrogen receptor hormone binding domain (FLP-EBD) fusion proteins in yeast and screening mutated ligand binding domains for altered ligand interaction. Having developed an effective strategy, I have applied it to (a) mutagenic library screening, and (b) ligand changes in yeast colony-color as a simple way to classify estrogen hormones and antihormones, and mutations that specifically affect ligand interaction. Using data from a number of mutations, I have found that the ligand induced positions of the ligand binding domain helix 12 and the F domain differentiate hormones and antihormones. This surely influences coactivator and corepressor interactions with ER in transcription because coactivator binding sites are formed with helix 12 (7,10). I also include work with the medically important antihormones, tamoxifen and raloxifene and have found one mutation in helix 12 that affects raloxifene specifically. These results very nicely coincide with the published structure of the ER LBD bound by estradiol or raloxifene (7). Though the two structures are static pictures, the conclusions one can draw from them match results from the recombinase assay of ligand activation presented here.

Results:

Screening of ligand binding as reported by recombination.

To regulate the FLP recombinase in yeast, the human ER hormone binding domain (domains D, E, and F; aa 251-595) was fused to the C-terminus of the entire coding sequence (423 aa) of FLP recombinase. The fusion gene was cloned under the control of the GAL10 galactose promoter (Figure 1A). Thus transcription and expression is limited to galactose media, with virtually no expression in glucose media. The fusion gene was inserted into a derivative of pRS315 (43), a single-copy CEN plasmid with the LEU2 selectable marker. Various restriction sites have been introduced into the estrogen LBD coding sequence (aa 306-595 of the human ER) without changing the amino acid sequence to simplify mutated library cloning.

To report FLP recombinase activity, a single-copy deletion recombination substrate was integrated at the TRP1 locus in yeast. The recombination target includes the constitutive alcohol dehydrogenase (ADH1) promoter directing transcription of the URA3 gene, followed by a poly-adenine signal to terminate RNA Polymerase II transcription (Figure 1B). The URA3 gene and a SUP11 ochre suppressor tRNA gene are flanked by FLP recombination targets (FRTs). The URA3$^+$ gene can be positively or negatively selected for growth. The SUP11 ochre suppressor tRNA gene between the FRTs allows a visual screen for recombination, using the red/white Ade2$^+$ colony color assay (32), as the tRNA suppresses the ade2-1 ochre allele and gives white colonies. A red pigment accumulates in ade2- cells. Thus recombination is detected by colony color assays or by Southern analysis.

Time course experiments confirmed the galactose control of expression was operating as designed, giving linear recombination between 4 and 10 hours, dependent on galactose for transcription and hormone to derepress the FLP-EBD protein (33). Hormone concentration experiments with a variety of known estrogen hormones and antihormones confirmed that the response of FLP-EBDs to ligands is a simple reflection of ligand binding by the EBD (33). All ligands tested (Fig. 1C), whether hormones or antihormones, induce the FLP-EBD fusion proteins.

Hormones and antihormones can be differentiated in yeast.

The D domain is a flexible, unstructured string of amino acids between the conserved C (DNA binding) and E (ligand binding) domains (6,13,20,38,42,49). We performed careful southern analysis of net FLP recombination and observed that constructs without a D domain were not activated by antihormones, even at very high concentrations (33).

An Ade2$^+$ color plate assay for ligand induced recombination was developed as a way to visualize the presence and concentration of ligands (34). We further tested the FLP-EBD with and without the D domain in the plate assay and confirm that antagonists (Z-OHT, Ral, Tam) as a class, do not activate FLP-EBD without the D domain spacer (Fig. 2, compare WT251 vs. WT304, where 53 amino acids of D domain have been deleted).

It was possible that the shorter form of the fusion proteins, FLP-EBD$_{304}$, failed to respond to antihormones due to a specific loss of binding

affinity. To address this possibility, we performed *in vitro* ligand binding experiments to measure estradiol and 4-hydroxytamoxifen binding by the FLP-EBD$_{251}$ and FLP-EBD$_{304}$ fusion proteins expressed in yeast (Fig. 3). Binding experiments utilized a fixed concentration of radiolabelled ^{3}H-estradiol (1nM), which was pre-mixed with zero or increasing amounts of unlabelled estradiol (E2; 1nM to 1000nM) or 4-hydroxytamoxifen (Z-OHT; 10nM to 10,000nM). We found that the FLP-EBD$_{304}$ form had the same binding affinity (half maximal inhibition, IC$_{50}$) for 4-hydroxytamoxifen (Z-OHT) and estradiol (E2) as the FLP-EBD$_{251}$ form (Fig. 3). Therefore if the antihormone concentration is sufficient to bind and induce recombination by the FLP-EBD$_{251}$ form, it should also activate the FLP-EBD$_{304}$ form, yet no induced recombination was seen, even with 10-100x more antihormone added (Fig. 2; ref. 33). We conclude that the *antihormone* bound FLP-EBD$_{304}$ protein *in vivo* is not capable of recombination due to steric hindrance with FLP, though the hormone bound FLP-EBD$_{304}$ is properly folded and active.

To verify that the fusion proteins were not responding differently for trivial reasons such as decreased protein stability, Western assays were performed (Fig. 3). Extracts of yeast expressing the various FLP-EBDs were prepared and compared with polyclonal antibodies to the last 16 amino acids at the C-terminus of ER (F domain). FLP recombinase/ ER fusion proteins migrate at the expected sizes, and two representative mutant forms of FLP-EBD do not change the amount or the size of protein recovered, implying similar expression and protein stability.

Antagonists can bind but not activate FLP-E/F estrogen receptor fusion proteins.

As shown before, a FLP-ER fusion protein that included the ER D, E and F domains (FLP-D/E/F) responded to all ligands tested, whether agonist or antagonist (Fig. 2, WT251; 33). This demonstrates that both ligand classes release ER from its initially repressed state and that the known differences between their activities are caused by differing specificities later in the pathway of transcriptional activation.

In contrast, the FLP-E/F protein, which has no D domain, was activated by agonists but not by antagonists (Fig 2, WT304; 33). This was unexpected since all studies with nuclear receptors show that the E domain is a modular entity that entirely encompasses the function of ligand

binding (50). To determine molecular reasons for the differential activity of these two classes of ligands, we constructed a number of mutation variants of FLP-ER involving the D, E, and F domains (outlined in Fig. 4 and discussed below). Consistent with previous studies, binding experiments using yeast extracts containing FLP-E/F and FLP-D/E/F proteins showed that both fusion proteins bound all agonists and antagonists with affinities close to those of the native estrogen receptor (33,34). The half maximal inhibition values for various ligands versus - labeled estradiol, are diagrammed next to the corresponding EBD form (Fig. 4). Hence lack of activation of FLP-E/F by antagonists was not due to a lack of binding.

F domain interference is a component of agonist and antagonist differences

Insight into the molecular basis underlying this difference between binding and activation was found from experiments that examined the role of the estrogen receptor F domain. In contrast to the FLP-E/F fusion protein which was not activated at all by antagonists, the FLP-E fusion protein, derived from FLP-E/F by deletion of the F domain, was partially activated by Z-OHT (Fig. 5A: compare FLP-E/F and FLP-E). Similarly, with FLP-D/E, the absence of the F domain also improved activation by Z-OHT (34). These results demonstrate that the difference between antagonist binding and activation was due in part to interference by the F domain. This interference was increased by removal of the D domain, resulting in E and F domains closer to FLP and its tetrameric reaction intermediate (see Fig. 6; ref. 8). In contrast, the proper, agonist bound conformation did not result in F domain interference (Figure 6, 7B).

Helix 12 mutation causes abnormal F domain interference with agonist binding

The x-ray crystal structures of the unliganded RXR and agonist-bound RAR E domains led to a "mouse trap" model of ligand binding by nuclear receptors, which invokes a large repositioning of the C-terminus of the E domain, helix 12 (6,38). It is important to note that no x-ray structures of ligand binding domains have included the F domain. F domain interference upon antagonist binding could reflect different positioning of helix 12, and consequently the F domain. If so, then altering the position of helix 12 by

mutation should invoke F domain interference upon agonist binding. This proved to be the case. Regardless of the inclusion of the "spacer" D domain or not, mutational disruption of helix 12 by proline (L540P) impaired agonist activation (Fig. 5A: FLP-E(L540P)/F; 34). This impairment was due in part to F domain interference, since deletion of the F domain from the helix 12 mutant protein restored activation (Fig. 5A: FLP-E(L540P)).

Clustered mutations of the EBD generated by codon substitution mutagenesis (CSM).

CSM, an oligonucleotide based method, is the best method for saturation mutagenesis of a region (9). The first mutation libraries focused on a region (aa 506-532; Fig. 8) previously implicated by random single mutation data to be important for estrogen ligand specificity. We have since generated libraries covering many of the predicted regions of importance for ligand interaction or specificity, as judged by the existing x-ray crystal structures of the retinoid receptors, RAR, RXR, and TR (6,38,49). A major improvement over the strategy outlined in the original proposal was to PCR amplify the synthesized oligonucleotide library and clone to unique sites of choice. This allows two advantages: (i) the inherently low chemical yields of long, mutated oligonucleotides are circumvented by PCR amplification, and (ii) subsequent cloning relies on only one unique restriction site in each region, which we have set up throughout the LBD. We have also made numerous site-specific mutations to test particular questions relating to hormone/antihormone function (see below; Tables I, II).

Screening assay for altered ligand binding specificity.

After generating a library of mutagenized FLP-EBD constructs, we perform a screening assay to enrich for plasmids containing mutations that are unreactive with a first ligand, yet still retain binding to a second ligand, resulting in recombination (as highlighted in Fig. 9). These plasmids are retested in the parent strain to confirm differential inducibility, as compared to the wild-type sequence, by a set of ligands. Screening of the libraries was useful in finding ligand inversion mutations, discussed below. Another type of mutation leads to a super induced phenotype, where the mutated form is more responsive to ligands than the wild-type sequence

(Fig. 9C, #6), and in this case leads to a slight raloxifene induction also. Examples are shown, using the plate assay, for the range of phenotypes between hormone and antihormone character (Fig. 9C). Table II summarizes a number of alleles sequenced with characterized phenotype. Mutations that respond as well as the wild-type sequence have amino acid changes which are very conservative. "Weak wild-type" phenotypes are still activated by agonists only, but with much reduced recombination activity. Some of these may be ligand affinity mutations. Other mutations (denoted wt+) are activated by a wider spectrum of ligands than the wild-type sequence. Altered specificity mutations show a drop or increase in activation by only one or several ligands. Finally a number of mutations block ligand binding to the receptor and do not derepress recombinase activity. These include non-conservative and multiple substitutions, as well as premature terminations within the LBD. A list of mutations throughout much of the LBD is shown in Table I, where fs = frameshifts and Term = termination codons.

Genetic selection of mutants depends on the positions of helix 12 and the F domain

In experiments to determine why the FLP-E/F protein was resistant to activation by antagonists, we used the connection between recombination and ligand activation to select mutants that switched from agonist activation/antagonist resistance to antagonist activation/agonist resistance. The selection procedure is outlined in Fig. 9A. FLP-E/F was mutated between amino acids 506 and 527 by codon substitution mutagenesis (9,14). This region was chosen because it includes helix 11 (Fig.8), which is important in agonist contacts in the RAR and TR crystal structures (38,49), and ER amino acid 521, the site of ligand selective ER mutants (11). FLP-E/F mutations that were resistant to activation by estradiol were selected by culturing the mutant libraries in the presence of estradiol while selecting for expression of the URA3 gene and hence, against recombination. Survivors were then cultured in the presence of an antagonist (Z-OHT or raloxifene) without selection, to permit growth after recombination, and then spread on plates to identify single recombinants by a red colony color. Red colonies identify EBDs with mutations between amino acids 506 and 527 that are resistant to activation by estradiol but

are activated by the antagonists Z-OHT or raloxifene. Candidate FLP-mutant E/F expression plasmids were isolated from red colonies and retested in the recombination reporter host (Fig. 1B) by a colony color assay. Yeast carrying the candidate expression plasmid and the unrecombined recombination reporter were spread as a lawn on a plate to which ER ligands were applied (Fig. 9B). The diameter of the circle of red yeast, induced by diffusing ligand, directly reflects the relative activation of the recombinase fusion by that ligand. Here the plate assay shows the agonist-only activation phenotype of the parent FLP-E/F protein, and for one of the mutants isolated (FLP-508), it shows the inversion phenotype of resistance to agonists, activation by antagonists.

Unexpectedly, no helix 11 mutations were recovered in this screen (Table III), although 17 independent mutations were identified (Table I). As a control, the known estradiol-resistant, antagonist-inducible G521R helix 11 mutation (11) was tested directly as a FLP-E(G521R)/F protein and was virtually inactive in this protocol (34). We attribute this to the fact that the G521R mutation is both a loss of binding mutation for estradiol (more than 10,000 fold) and a loss of binding mutation for Z-OHT (approximately 100 fold; 34) therefore making it too insensitive to ligands to be identified by this analysis. Testing the G521R mutation in the FLP-D/E/F background showed the expected increase in recombinase activity with less F domain interference. Most of the mutations identified affected helix 10 by introducing charged or polar residues into a very hydrophobic part of the α-helix (Table III). Southern experiments for other examples of these inversion mutants, exhibit good activation by antagonists and loss of agonist induction as expected from the plate assays (not shown). None of these mutants showed substantial differences in binding either agonists or antagonists. These mutations therefore affected estradiol activation, not binding, and so were conceptually similar to the case of antagonists with FLP-E/F (Fig. 5A). Consequently we tested a representative helix 10 mutation to establish whether the F domain was also responsible for interfering with activation.

FLP-E(L508R)/F was partially activated by Z-OHT and not by estradiol (Fig. 5B) as expected from the mutagenesis and the selection protocol used. Activation by both agonists and antagonists was improved when the F domain was deleted (Fig. 5B, FLP-E(L508R)). Abolishing helix 12 by

mutation had a slight effect on estradiol activation in the presence of the F domain (Fig. 5B: compare FLP-E(L508R)/F with FLP-E(L508R, L540P)/F). However the combination of the helix 12 mutation with the removal of the F domain fully restored estradiol activation (Fig. 5B, FLP-E(L508R,L540P)). Plate assay experiments, like those shown in Fig. 9B, 9C, also confirm the results and conclusions drawn from these southern assays (data not shown). Since none of these mutations had any significant effect on agonist or antagonist binding (Fig. 4), this demonstrates that the helix 10 mutations induced interference on estradiol activation by structurally repositioning helix 12 and the F domain.

Raloxifene activation is blocked by a mutation in Helix 12.

Repositioning helix 12 leads to a key determinant of ligand interpretation. In our studies relating agonists/ antagonists, we noticed mutations that specifically altered the response to raloxifene only. Plate assays using the D domain containing, WT251 form show that all ligands activate, and that deletion of the F domain only very slightly increases the ligand induced recombination (Fig. 10). Any form that includes disruption of the helix 12 (L540P) also specifically blocks any activation by raloxifene (Fig. 10). The region at helix 12 of the LBD is the critical helix for AF-2 function in transcription (7,10) and disrupts coactivators that bind to steroid hormone receptors (16,41). This is a key observation to understand the differences between tamoxifen and raloxifene types of antihormones.

Results in mammalian cells mirror those from yeast experiments.

One question of the yeast experiments was to understand how general the results would be if compared in mammalian cell experiments. The FLP-EBD recombinase system was set up in 293 kidney cells, using a similar single-copy integrated target, the LacZ gene, and an expression vector for the fusion recombinase (23). In almost all ways, results were directly comparable to those described from the yeast experiments. The fusion proteins maintain similar stability in western blots (not shown). The D domain was required to allow activation by estrogen antihormones; its removal left only hormone activation of FLP-EBD activity. The previously described mutation in helix 11, G521R (11), allows activation only by

antagonists and not agonists, both in transcription and in these FLP-EBD recombinase assays, further demonstrating the comparability (23). Interestingly, mutations at the N-terminal part of helix 10 that are thought to interrupt dimerization do not cause the inversion phenotype described above (23). This argues again that the inversion phenotype results from disrupted helix 12 and F domain position, not a block in receptor dimerization. Similarly, published transcription data in mammalian cells for mutations in helix 12 of ER have also confirmed an inversion phenotype with respect to activation by hormone or antihormones in transcription (see discussion; 24,29,30).

Discussion:

Activation with bound antihormones requires more distance between FLP and the estrogen LBD.

Previous work on several steroid receptors has implied that different conformations arise after hormone vs. antihormone binding. These conclusions primarily derive from protease clipping assays of the entire ligand binding domain, some 240 amino acids, and are necessarily imprecise (1). Others have shown differences in total hydrophobicity of the domain. In previous work with FLP-steroid receptor fusion proteins, we observed that all ligands, whether agonists or antagonists, served to release the cognate FLP-fusion protein from its initially repressed condition. This is true both in yeast (33) and mammalian cells (23). Therefore release from the initially repressed condition is concomitant with ligand binding and any differences in activities between agonists and antagonists must lie downstream. However we also observed that estrogen receptor fusion proteins that omitted the D domain, FLP-E/Fs, were activated by agonists but not by antagonists, in spite of the fact that both ligand classes were bound with near wild type affinities (33,34).

Different positioning of the LBD helix 12 and the F domain of ER accounts for functional differences between agonists and antagonists

In this report, we describe work to understand the observed difference between agonist/antagonist binding and activation. We found that antagonist activation can be partially restored by removal of the F domain, arguing that antagonist binding positions the F domain so that it interferes with FLP recombination. Repositioning the F domain by mutating helix 12 resulted in F domain interference with agonist binding. Genetically selected mutations that inverted agonist activation/antagonist resistance to agonist resistance/antagonist activation were similarly dependent on the positioning of helix 12 and the F domain. This gives new insight to the mechanism of similar phenotypic mutations for several nuclear receptors described in the literature, which had no previous explanation (see below). Thus three aspects important to the difference between ligand binding and activation were identified - the presence of the

F domain, the positioning of helix 12 and the proximity of domain E to FLP recombinase in the fusion protein.

Helix 10 causes the inversion phenotype by misplacing helix 12 and the F domain.

Taken together, the data can be explained by steric differences induced by binding of agonists or antagonists, or the mutations employed. In particular, we show that distancing domains E and F from FLP in the fusion protein by including the D domain in between dilutes interference in all cases. This discounts simple explanations that rely on intermolecular interactions with other components present in yeast and strongly favors intramolecular interactions within the fusion protein, reflecting steric differences in the estrogen receptor moiety of the fusion proteins. Figures 6 & 7 present a simplified explanation of the steric differences observed. In the case of the wild-type estrogen receptor, agonist binding positions helix 12, and consequently the F domain, in an organized conformation for cofactor binding and transcription activation by AF-2 (Fig. 11). Mutating helix 12 (L540P) disorganizes the positioning of helix 12 and the F domain, causing interference. Interference is also caused by antagonists bound to the wild-type estrogen receptor, an effect that is partly relieved by deletion of the F domain. Helix 10 mutations (at 508) disorganize the positioning of helix 12 and the F domain upon agonist binding yet permit, in part, an organized positioning of helix 12 and the F domain upon antagonist binding, leading to activation (Fig. 5B). Deletion of the F domain relieves interference in all cases (Fig. 5).

Antihormones are much larger molecules than hormones and probably interrupt the relatively compact LBD structure that would form around bound hormones (38,49), consistent with the new ER structures (7; Figure 12). We reason that the antihormone-induced conformation of the EBD interferes with the FLP reaction, as it does with cofactor binding and transcription activation (AF-2) in the native receptor (Fig. 11). We favor a model based on an organized positioning of helix 12 and the F domain by agonist binding. Previous work on ligand binding by nuclear receptors has implicated the correct positioning of helix 12 in agonist action (6,10,16,24,38). Our work extends these implications to demonstrate that the different positions of helix 12 adopted upon agonist or antagonist

binding can have a dominant effect in a functional enzymatic assay. Our work also highlights a role for the natural F domain in the differences between agonist and antagonist action, whereas to get x-ray structures has required deletion of the F domain.

Since AF-2 activity is not transcriptionally measurable in yeast, this FLP assay is a first for discrimination between hormones and antihormones at a structural level in yeast. For example, a compound which induces recombination with the FLP-D/E/F form but not the FLP-E/F form likely has antihormone properties and can be tested in full-size ER assays for confirmation.

The crystal structures of the ER LBD confirms helix 12 (and F domain) positions.

During the past year, the x-ray crystal structures of the estrogen receptor hormone binding domain bound by estradiol or raloxifene were published (7). The structures show a very large repositioning of the helix 12 by raloxifene, relative to the estradiol bound form (Figure 12). Hence our data confirms this aspect of the x-ray structures with functional data, and the x-ray structures confirm our basic conclusions. In addition, the F domain position, though not included in the x-ray structures for technical reasons, would be vastly different when the ER LBD is bound by raloxifene instead of estradiol, as also inferred from the studies presented here. The helix 10 inversion mutations identified here occur along the dimerization interface, though the exact position of 508 and other helix 10 residues was not presented in the crystal structures. Helix 10 and 11 are contiguous in the ER LBD and are simply referred to as helix 11 in the recent structure, (7). It is plausible that the helix 10 mutations we identified alter dimerization in such a way as to relax or alter the steric positioning of the ER LBD with respect to the FLP recombinase. However secondary alterations to the overall conformation of the ER LBD, independent of effects on dimerization, could also explain, or contribute to, the inversion phenotype observed. Finally it is worth noting that mutations that impair dimerization would also impair transactivation in a conventional transcription assay and would not distinguish between agonists and antagonists. The non-transcriptional basis of the assay employed here

permitted a description of agonist- and antagonist-specific steric differences.

One report of potential linkage of an ER allele and breast cancer has been recently reported (44). In this case, the codon change at amino acid 325 does not change the amino acid from the wild-type proline (Fig. 8). It also does not occur near any of the splicing signals, as it is central to exon 4 of ER (see Fig. 7A). Hence it is not surprising that the authors found no correlation to breast cancer incidence with that allele, as no functional differences in ER protein would be predicted.

Inversion mutations of other nuclear receptors.

Several studies based on transcriptional assays have identified mutations in ER, and other steroid receptors, that convert antagonists into agonists (19,24,29,30,48). All of these mutations occur near to or in helix 12 and can, in the context of the work presented here, be evaluated as conformational mutations that disorder helix 12 and reposition the F domain. In one study, the ER helix 12 mutation, L540Q, inverted transcriptional activity so that antagonists induced activity but estradiol did not. Deletion of the F domain from the L540Q mutant ER restored estradiol-induced, and diminished antagonist-induced, transcriptional activity (30). This indicates that, by this mutation, the F domain is differently positioned upon agonist and antagonist binding in a transcriptional activity assay. Work on the glucocorticoid receptor (GR) mutation, I747T which lies between helices 11 and 12, documents a complementary observation of a difference between near normal binding of agonists and impaired transcriptional activity due to, we suggest, altered helix 12 position and F domain interference (39). We also suggest that different positioning of the F domain will differently affect co-factor binding. By analogy to the local steric effects on FLP recombination, we reason that a mispositioned helix 12 and F domain can interfere with local protein/protein interactions relevant to steroid receptor action. Of particular interest are interactions with steroid receptor transcriptional cofactors. Recent work has indicated that a variety of nuclear receptor cofactors exist (16;17 and references within; 41). It may be possible that different agonists and antagonists, by inducing different helix 12 and F domain positions, differently influence cofactor interactions. By this

means, selectivity among cofactor binding directed by different ligands may be achieved. We also note that the same considerations of local protein/protein interactions may also influence receptor homo- or heterodimerization. Further work is required to establish the degree to which these speculations describe the transcriptional activities of the steroid receptors.

The "F domain" length is conserved.

The F domain may not be as inert for steroid receptor function as previously thought. It has not been included in any x-ray structures so far, for technical reasons, and yet abuts helix 12, important for cofactor binding and ligand activated AF-2 function (10,16). An examination of F domains (defined here as all amino acids after helix 12, the last conserved sequence) from all available steroid receptor sequences revealed a striking observation - namely, the lengths of the F domains have been largely conserved (34). ERα has the longest F domain, 50 amino acids in all known higher vertebrates. Fish ERα F domains differ somewhat in that they are usually even longer, up to 84 a.a. All three known ERβ have F domains of 34 amino acids in length. Remarkably, all other steroid receptors extend exactly 19 amino acids after helix 12, with one exception (trout glucocorticoid receptor has 25). Since there is little sequence conservation, even within a single receptor type, the conservation of F domain lengths is very unlikely to be coincidental. We reason that there are functional constraints that bear upon the F domain and that one of these constraints, as identified here, is the organized positioning of the F domain upon agonist binding.

Hsp90 complex regulates LBD-fusion proteins.

Current models to explain LBD regulation of proteins to which they are fused give a primary role for the Hsp90 complex (36). The Hsp90 complex is ubiquitous and abundant, and possesses chaperonin activity (37). Further evidence that the steroid receptor LBDs are associated with this complex in the unliganded state has come from genetic experiments with yeast (5,18,31). Fusion of an LBD onto a heterologous protein is believed to direct the fusion protein to associate with the Hsp90 complex (40). Binding of agonists promotes LBD release from the complex, thus derepressing the fusion protein functions. Whether all antagonists serve to release LBDs from the Hsp90 complex to the same extent remains unclear.

Materials and Methods

Strains and chemicals. The *S. cerevisiae* strain used for these experiments (*MAT **a**, leu2-3,112, his3-11,15, ura3-52, trp1-1::(TRP1,URA3,SUP11), ade2-1ochre, can1-100)* was derived from RS453 (R. Serrano, Valencia, Spain) by integrating the target of recombination (Fig. 1A) at the *trp1* locus. Transformation of yeast by the standard lithium acetate method was performed as described (2). Transformed yeasts were grown and maintained with selection for leucine and tryptophan in glucose or galactose supplemented synthetic media from BIO 101, Inc. The hormones and antihormones were purchased from Sigma, except 4-hydroxytamoxifen (Research Biochemicals International), and ICI 182,780 (a gift from Dr. A. Wakeling, Zeneca Pharmaceuticals).

Southern assays and ligand titration experiments. Transformed yeast, containing the GAL10 promoter, FLP-ER gene on pRS315 (43), were grown in synthetic glucose medium lacking leucine and tryptophan to OD_{600} =1.5. Equal volumes of cultures were collected and resuspended in medium containing 2% galactose with or without ligands, which was dissolved in ethanol as a 1000 or 10,000 fold stock solution. The "no hormone" samples received an equal volume of ethanol. Cells were collected at times noted and DNA was prepared by standard procedures using a zymolyase 20T (ICN) incubation, SDS lysis, followed by potassium acetate precipitation as described (2). About 10 μg of DNA per lane was digested with PstI and loaded on 0.7% gels in 1x TAE buffer. Gels were treated with 0.25M HCl for 10 min, 0.4M NaOH for 2x 30 min, 20x SSC for 30 min, and then blotted to Qiagen nylon plus filters with 20x SSC. After baking the filter at 80°C for 2 hr, they were probed at 72°C with a riboprobe, made from the 1.2 kb ScaI-BsiWI fragment of the *E. coli* LacZ gene, in a buffer containing 250 mM sodium phosphate pH 7.2, 7% SDS, and 1 mM EDTA. Washes were performed in 25 mM sodium phosphate pH 7.2, 1% SDS and 1 mM EDTA at 72°C. Recombination was calculated as a ratio of [counts in the recombined band/(counts in recombined + unrecombined bands)] and was therefore not affected by minor variations in the amount of DNA loaded.

Ligand binding assay. Ligand binding experiments were performed to measure estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182,780

binding by the various FLP-ER fusion proteins. The protein extracts were made from FLP-ER transformed yeast, grown in galactose without hormones to OD_{600} = 1. The resuspended yeast pellets were lysed using a glass bead procedure (2) in a buffer containing 20mM Tris, pH 7.9, 10mM $MgCl_2$, 1mM EDTA, 5% glycerol, 1mM DTT, 420mM KCl and protease inhibitors. The ligand binding experiments were done in 300 µl volume with a fixed concentration (1nM) of radiolabelled ^{3}H-estradiol (84 Ci/mmol; Dupont NEN), which was pre-mixed in the respective tubes with zero or increasing amounts of unlabelled ligand (1nM to 1000nM). The binding was at 4°C for 16-18 hours in buffer (PMMG) containing 8.5mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.5, 10mM sodium molybdate, 2mM monothioglycerol, 20% glycerol and 1 mg/ml protein extract. After the binding incubation, unbound label was absorbed by adding 300µl of DCC (0.5% charcoal Norit-A, 0.05% dextran T70) in PMMG buffer for 15 min at 4°C and then centrifuging at 12,000 rpm for 5 min. Equal volumes of supernatant were quantified by liquid scintillation counting. Binding values (IC_{50}) are expressed as the amount of unlabelled ligand competitor needed to reduce to 50% the ^{3}H-estradiol bound in the absence of unlabelled steroid.

Color plate assay. Yeast containing the integrated SUP11 recombination substrate (Fig. 1B) were transformed by plasmids containing the various FLP-ER genes. Cultures were grown in glucose with leucine selection and then plated at high density on synthetic galactose plates lacking leucine and tryptophan. A 2 µl drop of ethanol containing each ligand was placed on the plate, as shown (Figs. 2, 9B, 9C, 10). An adenine marker in yeast gives a color phenotype (red) if the target gene has been deleted by the recombinase. The plates were grown at 30°C for 4 days to maximize red color formation.

Generation of mutated FLP-ER libraries by codon substitution mutagenesis (CSM)

CSM was performed as described (9) on amino acids of the human estrogen receptor (outlined previously).

CONCLUSIONS

The properties of estrogen ligand-inducible recombination mediated by FLP-EBD fusion proteins show that all ligands activate FLP-EBD fusion proteins with a D domain, yet without, antihormone binding forms an EBD conformation which blocks recombination by a steric mechanism, emanating from a misaligned helix 12 and F domain. This presents a simple assay to predict hormone vs. antihormone activity of a compound in yeast, as well as a way to screen for functional interactions with amino acids defining hormones vs. antihormones. We have used codon substitution mutagenesis (CSM) to generate mutagenized libraries. Estrogen induced changes in yeast colony color has been used as a simple method to detect ligand binding, and to measure its relative hormone and antihormone character. There has been a change of focus from exclusively ligand binding changes to include those mutations that cause functional differences between agonists/ antagonists. We have addressed this with work demonstrating helix 12 and F domain importance. The FLP-EBD fusions exhibit almost identical properties in mammalian cells as in yeast (23), and we have characterized numerous mutations in the EBD (Table I).

Key amino acid determinants of ligand binding in the estrogen receptor have been found at E353, R394, H524, and other amino acids in helices 3, 10 and 11, as confirmed by the ER LBD structures (Fig. 12; 7). Do FLP-EBD fusion proteins define functional components of hormone versus antihormone action? We have made good progress toward understanding components of the LBD that participate in antihormone blockage of ER, namely helix 12 and the F domain. Many of the mutations we have studied have generated altered LBD structures, as opposed to ligand affinity changes. Other mutations clearly have an affect on one or several ligands, such as D351, H524 and L540. With the progress made during these studies and the very significant x-ray structures of the ER LBD, there is now a better understanding of how tamoxifen and raloxifene bring about tissue-specific effects. Clearly each ligand presents a different shaped binding surface on the LBD for the specific cofactor interactions. When those surfaces are perturbed, cofactor binding drops to a fraction of normal, leaving transcription by ER to the AF-1 domain alone. With this more precise ligand pocket definition, a goal to develop better therapeutic agents, stemming from the new molecular information may be realized.

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Figure Legends

Figure 1. (A) Diagram of the FLP-EBD expression plasmid displaying its functional elements. The gene for the FLP-ER LBD fusion protein is driven by a galactose promoter on a plasmid in yeast. **(B) Diagram of the FLP recombination deletion strategy.** Before recombination, the constitutively active ADH1 promoter expresses the URA3 selectable marker which lies between directly repeated FLP recombination targets (FRTs, shown as triangles). The polyadenylation signals (pA) after URA3 prevent LacZ expression. Also lying between the recombination targets is a SUP11 gene which is transcribed in the opposite direction, as depicted by the short arrow. Downstream of the second recombination target is the LacZ coding region. After recombination, the URA3/SUP11 region is excised and the LacZ gene juxtaposed to the ADH1 promoter. Recombination mediated alterations in cellular phenotype are displayed at the right of the diagram. Note that expression of the endogenous Ade2+ gene relies on SUP11 expression. The diagram also outlines the Southern strategy employed. A 5.6 kb fragment is reduced by recombination to a 4 kb fragment when a probe from the LacZ gene is used. **(C) Structures of the ligands tested with the FLP-EBD.** The hormones are shown above the line, antihormones below.

Figure 2. Color plate assay, comparing the effect of the D domain with activation by antagonists. FLP-EBD recombination in a color plate assay in yeast reflects affinity and the concentration of a hormone placed on a lawn of unrecombined yeast cells. Yeast containing the SUP11 recombination substrate (Fig. 1) and either the WT251 (FLP-D/E/F) or WT304 (FLP-E/F) fusion proteins were plated at high density onto low adenine medium in a standard 9 cm petri dish. As is apparent, the D domain selectively alters ligand responsiveness. Deletion of the D domain (WT304) renders the fusion recombinase insensitive to any of the antihormones tested, as opposed to the FLP-EBD$_{wt}$ (WT251) control. An adenine marker in yeast gives a color phenotype (red) if the target gene has been deleted by the recombinase. A 1 µl drop of ethanol containing the ligands were plated on the yeast lawn and grown for several days.

Figure 3. Ligand binding and Western blots of FLP-EBD proteins. The top panel shows the results of *in vitro* ligand binding competitions with the FLP-EBD$_{251}$ and the FLP-EBD$_{304}$ forms. Labeled ^{3}H-estradiol (1nM) was bound without or with increasing amounts of unlabelled estradiol (E2) or 4-hydroxytamoxifen (Z-OHT). The 100% binding points are determined in the absence of added cold ligand, minus background (marked NL). The lower panel shows the results of western blot experiments, using an antibody to the C-terminal, aa 576-595 of ER. The natural human ER is present at 66kDa in MCF-7 cells. FLP recombinase/ ER fusion proteins are larger; the WT251 fusion form is 88kDa and the WT304 form is 82kDa. Two mutated forms of the EBD (251-17, 304-17) do not change the steady-state amount or the size of protein recovered from yeast.

Figure 4. Schematic representation of various forms of the estrogen receptor fused to FLP, with ligand affinities for each. In these constructs, the D domain starts with hER amino acid 251, the E domain with a.a. 304 and the F domain deletion omits a.a. 552-595 (see Figs. 7A,8). Positions of the helix 10 mutation (L508R) and the helix 12 mutation (L540P) near the C-terminal end of the E domain are also shown as asterisks. IC$_{50}$, the amount of cold ligand competitor needed to reduce 1nM ^{3}H-estradiol bound in the absence of cold steroid to 50% is shown for each of the FLP-ER forms and ligands. None of the mutations or deletions shown significantly affect binding by the ligands. E2- estradiol, Z-OHT- 4-hydroxytamoxifen, RAL- raloxifene, ICI 182,780.

Figure 5. The activity of FLP-ER fusion recombinases is affected by the presence of the D and F domains, as well as helix 12 integrity. The figure shows southern blots used to measure recombinase activity in ligand titration experiments with either estradiol (E2) from 10^{-6} to 10^{-10} M or 4-hydroxytamoxifen (Z-OHT) from 10^{-5} to 10^{-9} M. Control samples were cultured in glucose (gl, first lane) or galactose without ligands (-). Panels A & B shows experiments with FLP-ERs without the D domain and: with or without the F domain (FLP-E/F vs. FLP-E); or including the L540P mutation with or without the F domain [FLP-E(L540P)/F vs. FLP-E(L540P)]. Panel B shows the equivalent FLP-ERs which contain the additional L508R "inversion" mutation.

Figure 6. FLP-EBD recombinases show activation by hormones and not by antihormones, due to a tetrameric intermediate. The intermediate for recombinase activity involves a tetramer of proteins (8). With an antagonist bound EBD, the recombinase intermediate is destabilized and inactive, only if the D domain is not part of the fusion. Only the correctly folded hormone bound form of the EBD allows recombination.

Figure 7. (A) Schematic diagram of the human ERα gene and protein. The conserved domains, A-F, are shown above the gene with the amino acid position numbers at each border. The C domain (zinc fingers for DNA binding) and E domain (ligand binding domain, responsible for trans-repression of FLP) are conserved among nuclear receptors. The exon structure is also shown with the amino acid positions at each intron-exon boundary. Many ER variants found previously miss one or more exons. **(B) Summary of the ligand induced activity of FLP-ER fusion proteins containing only the E and F domains.** The activity of FLP is very sensitive to proper folding of the ER domain after hormone binding. Mutations perturbing the fold will also affect induction of recombination (see Fig. 9B, 9C).

Figure 8. The protein sequence of the C, D, E, and F domains of human estrogen receptor. The fusion points used here for the FLP chimeras are signalled by the arrows at 251 (FLP-D/E/F) or 304 (FLP-E/F). The structural elements of the hormone binding domain, deduced from sequence alignments and the known ER structure (7), are mapped onto the sequence of the human ER. Boxes outline α -helices (H1 to H12) and arrows mark β-sheets (S1, S2). The positions of exon boundaries and the sizable F domain are also marked.

Figure 9. (A) Isolation of mutations that show altered specificity of ligand induced recombination. Libraries of mutagenized FLP-ER were grown in the presence of estradiol to activate FLP-ER recombination, and in the absence of uracil, to select against recombination. Thereby FLP-ER mutants not activated by estradiol were enriched. In step 2, the surviving cells were grown with a second ligand (Z-OHT) in the presence of uracil to permit recombination and then screened on plates for red colonies indicating recombination. Plasmids were rescreened to verify estradiol resistance and antagonist inducibility. This pool analysis helps to recover mutated forms with very low abundance.

(B) Colony color plate assay to evaluate ligand responsiveness of FLP-ERs from the screen in (A). The plasmids were retested in the parent strain to confirm differential inducibility by a set of ligands, as shown, in plate assays. FLP-E/F is activated only by agonists (E2, HEX, DES) and an "inversion" mutant (FLP-508) is activated only by antagonists (Z-OHT, RAL, TAM).

(C) Colony color plate assay to evaluate ligand responsiveness of various mutated FLP-ERs. The hormones and antihormones are plated in the same pattern as (B). The diameter of the circle of red yeast, induced by diffusing ligand, directly reflects the relative activation of the recombinase fusion by that ligand. The first plate, FLP-E/F is the control, with wild-type ER sequence. Each of the numbered variants shown is a single amino acid substitution that affects ligand interaction/ protein conformation of ER, fused to FLP. Numerous conservative amino acid substitutions showed no change from wild-type ER (not shown). The red color plate assay is quite sensitive and reproducible for a panel of ligands.

Figure 10. Plate assays show that a disruption mutation of helix 12 causes the singular loss of raloxifene activity. The plates shown have ligands placed as in Fig. 2 and Fig. 9B. Deletion of the F domain increases activity slightly, especially for the L540P, helix 12 disrupting mutation (right-hand panel).

Figure 11. Schematic diagram of the natural estrogen receptor in transcription. Ligand binding to ER releases the repressive Hsp90 complex, allows dimerization, and DNA binding to an ERE upstream of a target gene. This complex includes coactivators such as SRC1 and CBP, which stimulate RNA Polymerase II activity. Antihormones bind and misfold ER at helix 12 and the F domain, blocking the cofactor interaction that stimulates transcription.

Figure 12. Ligand binding pocket of ER is compared when estradiol vs. raloxifene is bound (from ref. 7). Most of the amino acid contacts are similar, yet raloxifene, an antihormone, blocks helix 12 from generating the coactivator binding site. Important amino acids for binding are highlighted and described from the included figure legend (7).

Table I. EBD mutations.

C 530 L
M 528 L
K 529 C
Term 527 am
fs 524, Term 526
fs 529 (term 538)
K 529 I
K 529 T

L 511 K
L 508 W, L 509 S, I 510 A
R 515 G
L 511 A
L 509 F, M 517 E, K 520 G, G 521 V
H 516 D, H 524 R, L 525 A, S 527 R

G 521 R
L 507 G, L 511 G, K 529 N, C 530 A
L 511 P
K 531 R
I 510 A, R 515 T
L 508 A, L 509 G, I 514 L, H 516 R
L 508 T, M 517 D
L 511 R, H 516 A
L 507 I, L 508 K, I 510 A, H 513 Y
I 510 P, L 511 P
Q 506 D, L 508 Y, I 510 L, L 511 R
L 509 S, I 510 A, L 511 D, R 515 G
fs 513, Term 518
fs 515 (Δ 13 bp)

K 520 T, G 521 C, E 523 R
H 516 V, M 517 P, M 522 A, L 525 T,Y 526 G
L 511 S
I 510 S, L 511 Term am (H 513 E)
Q 506 T, L 507 P, L 508 Term op
L 509 V
N 519 Term am

Q 314 P, Term 315 op

fs 425, Term 457

L507V, L508Q, L509W, I510S, L511 Term
L 508 Term op, Δ 23 bp
I 510 G
I 510 E, H 513 N
L 509 K, I 510 R, fs 519 Δ 4, Term op 527
L 509 R
Q 506 G, I 510 E, L 511 C
D H516, D M517 (Δ 6 bp)

I 514 Q
L 508 Term op, Δ 23 bp
L 509 D, R 515 K
L 508 R, L 509 H, L 511 G
L 508 W, I 510 T, fs 511 , Term 517
I 510 P, L 511 S, Δ 15 bp, M517 wt>
L 508 S, L 509 G, I 510 S, fs 511, 517 Term
fs 511, Term 519 och
L 508 E, I 510 R, R 515 C
L 507 K, L 508 A
L 507 A, L 509 T, S 512 D
Q 506 G, L 508 P, I 514 L
L 507 N, L 508 V
I 510 N, M 517 P
I 510 S, M 517 V
L 511 P, R 515 P
L 508 P, I 510 A, L 511 Term op
L 507 G, L 511 C, R 515 A
L 509 S, L 511 R
L 509 R, S 512 L, H 513 Term op
Q 506 L, L508H, L509R, I510E, M517 Term
R 515 G
L 508 H, I 510 L, I 514 T
I 510 A
S 512 G, M 522 L
G 521 Q, M 522 L, E 523 D, Y 526 G

L 507 M, L 508 G
H 524 Y
R 515 C, H 516 L
I 510 R
R 515 V
I 514 Q
L 507 I
L 508 R
L 508 R, L 540 P
L 507 G
L 507 Term

L 507 G, L 508 T
L 509 N, H 524 Y
L 508 I, S 512 E

L 508 D, L 509 R
L 509 D, R 515 K
L 507 V, L 508 K
L 508 R, L 509 D, M 517 Y
L 508 R, L 509 C, S 512 Q
L 508 R, L 509 A, L 511 T
L 507 V, L 508 Q, L 511 A, R 515 C

Table I. (continued)

Y 526 G
L 508 P, L 525 P
H 524 S, Y 526 R, S 527 G
H 516 D, N 519 F, K 520 T
N 519 T, E 523 I, H 524 Y

R 515 Q
G 521 A, E 523 R
I 510 P, L 511 G

L 508 K
L 508 T, R 515 L
L 507 D, I 514 G
L 508 G, L 509 I, R 515 G
L 508 S, L 509 A, S 512 V
L 507 V, L 508 G, L 509 S
L 508 H, I 510 L, I 514 T
L 508 D, L 509 R
L 509 R, D 510-511
L 511 R, M 517 I
L 508 R, M 517 L
L 508 H, R 515 K
I 510 S, S 518 N
L 509 Q, L 511 W, S 512 P, R 515 A
I 510 D, Δ 511-512, R 515 K
H 524 Y
L 509 R, S 512 G
D509, H 513 T, M 517 Y

L 508 K
L 508 G, L 509 I, R 515 G
L 508 E
L 508 H, I 510 L, I 514 T
L 508 Q
L 508 H, R 515 K
L 508 R, M 517 L
L 509 R, S 512 G
L 509 R, I 510 D, L 511 R
L 509 N, M 517 I
L 509 V

A 322 V, E 323 V
A 322 V
A 322 G, E 323 G
A 322 D, E 323 A
I 326 R, L 327 H
L 327 R
I 326 K, L 327 H
D 351 H, E 353 A
D 351 H
D 351 Y, E 353 V
R 352 G
D 351 K, R 352 G, E 353 A
N 359 S, W 360 R, K 362 T
W 360 G, K 362 T
W 360 R, K 362 T
W 360 R
W 360 G, K 362 R
W 360 G, K 362 R, R 363 G

L 508 D
fs 432, Term 454 ochre
S 432 V, S 433 T
M 427 T
M 437 H
L 429 Q
M 517 C, M 522 G, H 524 L, S 527 E

Term 406 och
S 395 P
A 382 K, L 384 P, I 386 Term op
H 398 L, fs 401, Term 405 och

H 398 Term amb
K 401 S, L 402 Q, F 404 W, P406L, N407M,
D 411 R, R 412 N, N 413 Q, Q 414 R

L 408 D, N 413 F, G 415 P, G 420 L
R 412 A, N 413 R, C 417 Q, V 418 L, E 419 E
419 A, G 420 A, M 421 S
F 461 Q, fs 463, Term 518
L 453 F, L 466 R
G 457 H
N 455 F, V 458 H, Y 459 P, T 460 F, S 464 V
M 427 K, S 432 C, R 436 Term amb
S 433 L, R 434 P, F 435 Y, R 436 G
A 430 E, Q 441 L

S 464 C
F 461 A
S 433 N
fs 431 (Δ1)

P 399 S, K 401 G, L 402 Term och
G 420 N
N 413 I, G 420 L, M 421 K
N 413 D, fs 416
D 411 Y, M 421 N
N 407 D, D 411 P, E 419 W, G 420 K
P 406 G, D 411 T, R 412 T, N 413 Q, Q 414 R
P 399 G, G 400 R, L 403 K, L410P, D411L,
R 412 N, N 413 Q, Q 414 R

Table I. (continued)

C 381 G, A 382 E
E 470 Q
L 466 A
Y 459 I, fs 464 (Δ1)
M 437 L
A 382 V, M 388 F, W 393 A
L 391 P
C 381 fs, 384 Term amb
A 382 fs, 391 Term opal
P 399 G, L 402 fs, Term > 416
K 401 G, L 409 A, R 412 N, N 413 Q, Q 414 R

L 453 G, G 457 Q
L 454 P, V458L, Δ461-464 (FLSS), T465P
S 456 D, G 457 V
I 451 T, Y 459 C, S 464 L
N 455 P, F 461 R, S 464 R, T 465 H, L 466 V
N 455 R
S 456 P, S 464 C
I 451 A, L 454 P, G 457 N, T 460 L
N 455 S, T 460 H, S 464 F

L 428 V, L 429 T, F 435 G
R 436 G, M 437 D
T 431 R
M 438 G
M 427 fs, M 437 Term opal
L 428 G, L 429 E, M 437 S

M 427 R, L 428 G
S 456 F, Y 459 P, L 462 K
I 451 A, L 454 P, G 457 N, T 460 L
N 455 S, Y 459 P, L 462 S, S 463 N
S 456 K, Y 459 S
N 455 S, Y 459 P, L 462 S, S 463 N
I 452 N, S 456 P, S 463 F

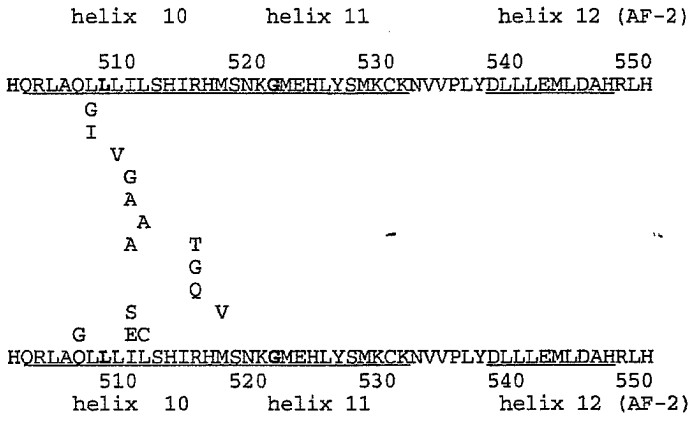
G 390 S, L 391 R
R 394 S
L 391 H, R 394 G

C 381 E, A 382 Term amb,
I 389 A, G 390 P, W 393 G

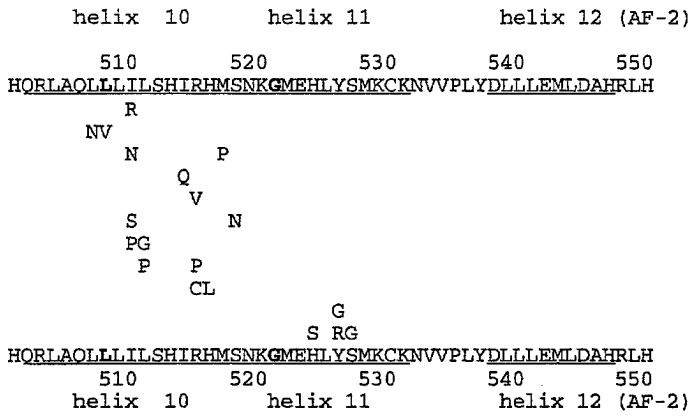
Table II.

Human Estrogen Receptor - LBD

Wild-Type



weak wt



WT +

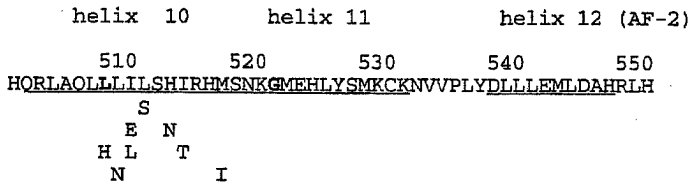


Table II (cont).

altered specificity

helix 10 helix 11 helix 12 (AF-2)

510 520 530 540 550
HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH

G
R G
GT
Y
N Y
A R
T IY

Inactive

helix 10 helix 11 helix 12 (AF-2)

510 520 530 540 550
HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH

MG
KA
R
R I
S R
T L
RDR
D G
G C A
G P L
GI G
E R C
TC R
Δ T Y
Δ FT
RΔΔ
Q WP A
Term
Term
Term
fs Term
fs Term
fs Term

HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH
510 520 530 540 550

Table III. Mutations recovered by selection for agonist insensitivity/antagonist activation are shown underneath the amino acid sequence of the C-terminus of the ER ligand binding domain, E. The positions of helices 10, 11 and 12 are indicated.

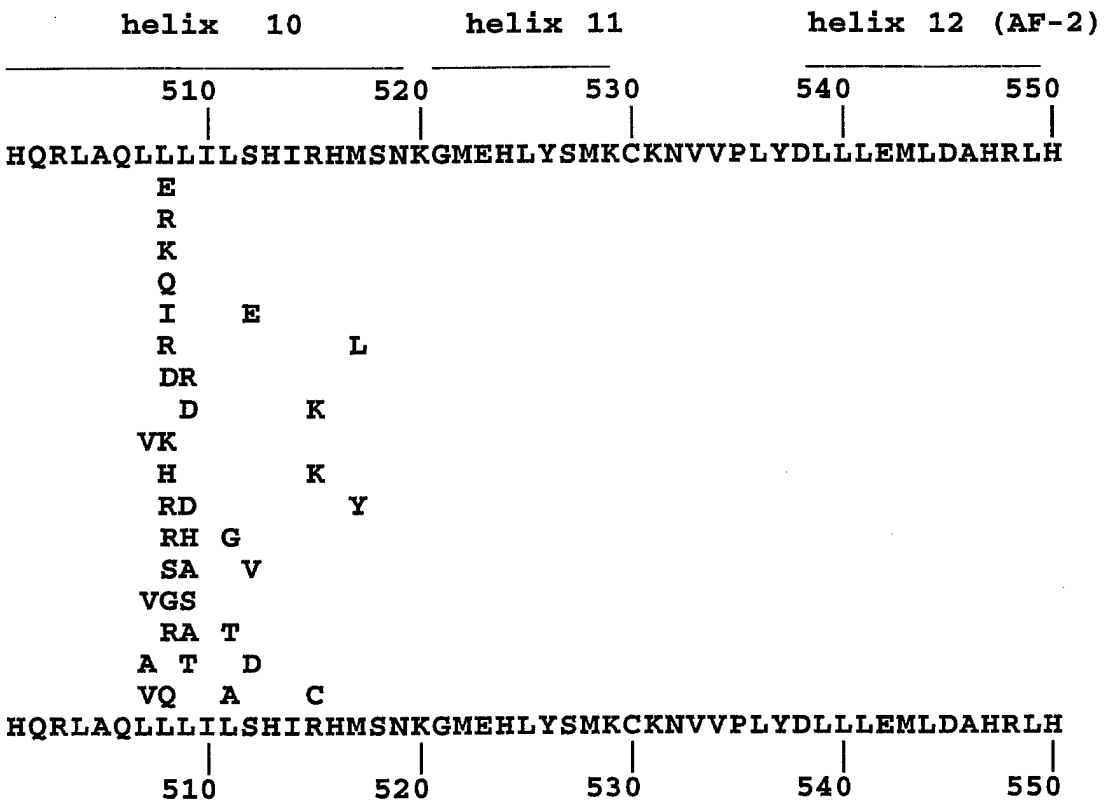


Figure 1.

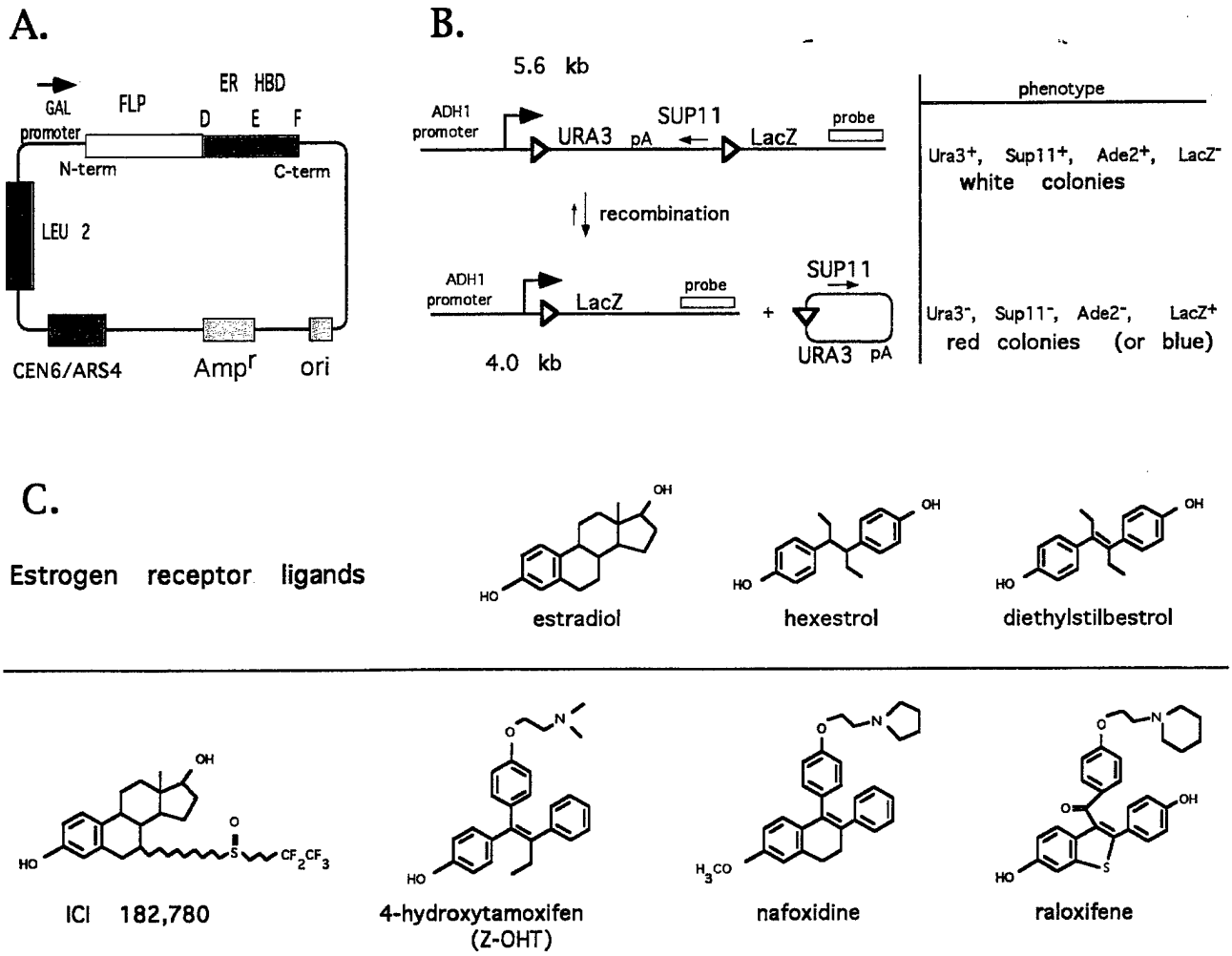
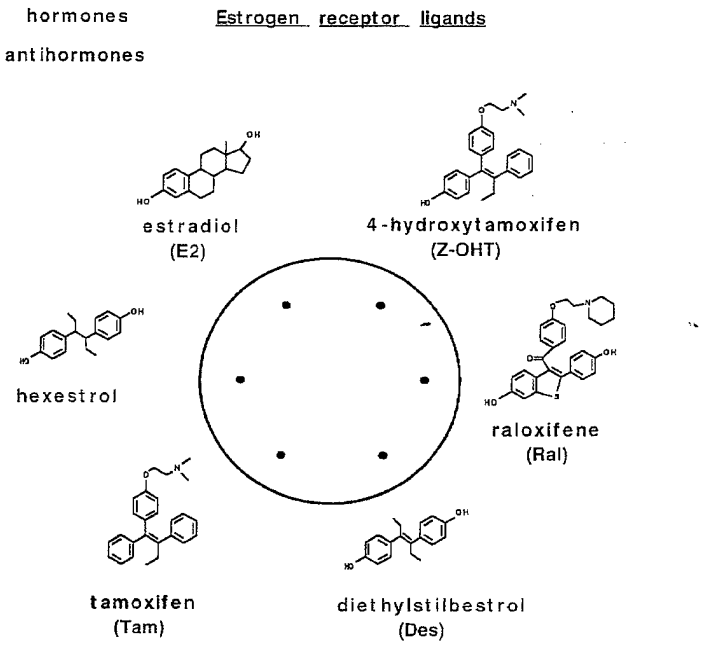

A.
GAL
promoter
FLP
ER HBD
D E F
N-term
C-term
LEU 2
CEN6/ARS4
Amp^r
ori
B.
5.6 kb
ADH1
promoter
URA3
pA
SUP11
LacZ
probe
recombination
ADH1
promoter
LacZ
probe
SUP11
URA3
pA
4.0 kb
phenotype
Ura3+, Sup11+, Ade2+, LacZ-
white colonies
Ura3-, Sup11-, Ade2-, LacZ+
red colonies (or blue)
C.
Estrogen receptor ligands
estradiol
hexestrol
diethylstilbestrol
ICI 182,780
4-hydroxytamoxifen
(Z-OHT)
nafoxidine
raloxifene

Figure 2.


hormones
antihormones
Estrogen receptor ligands
estradiol
(E2)
4-hydroxytamoxifen
(Z-OHT)
hexestrol
raloxifene
(Ral)
tamoxifen
(Tam)
diethylstilbestrol
(Des)

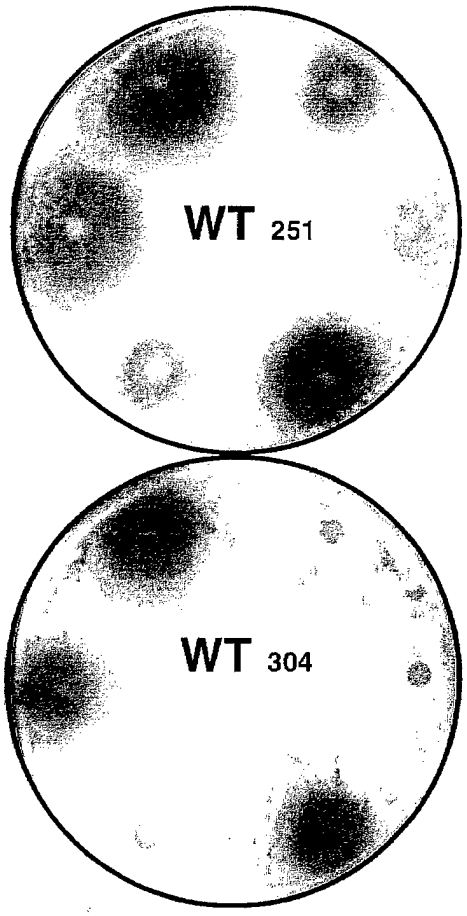
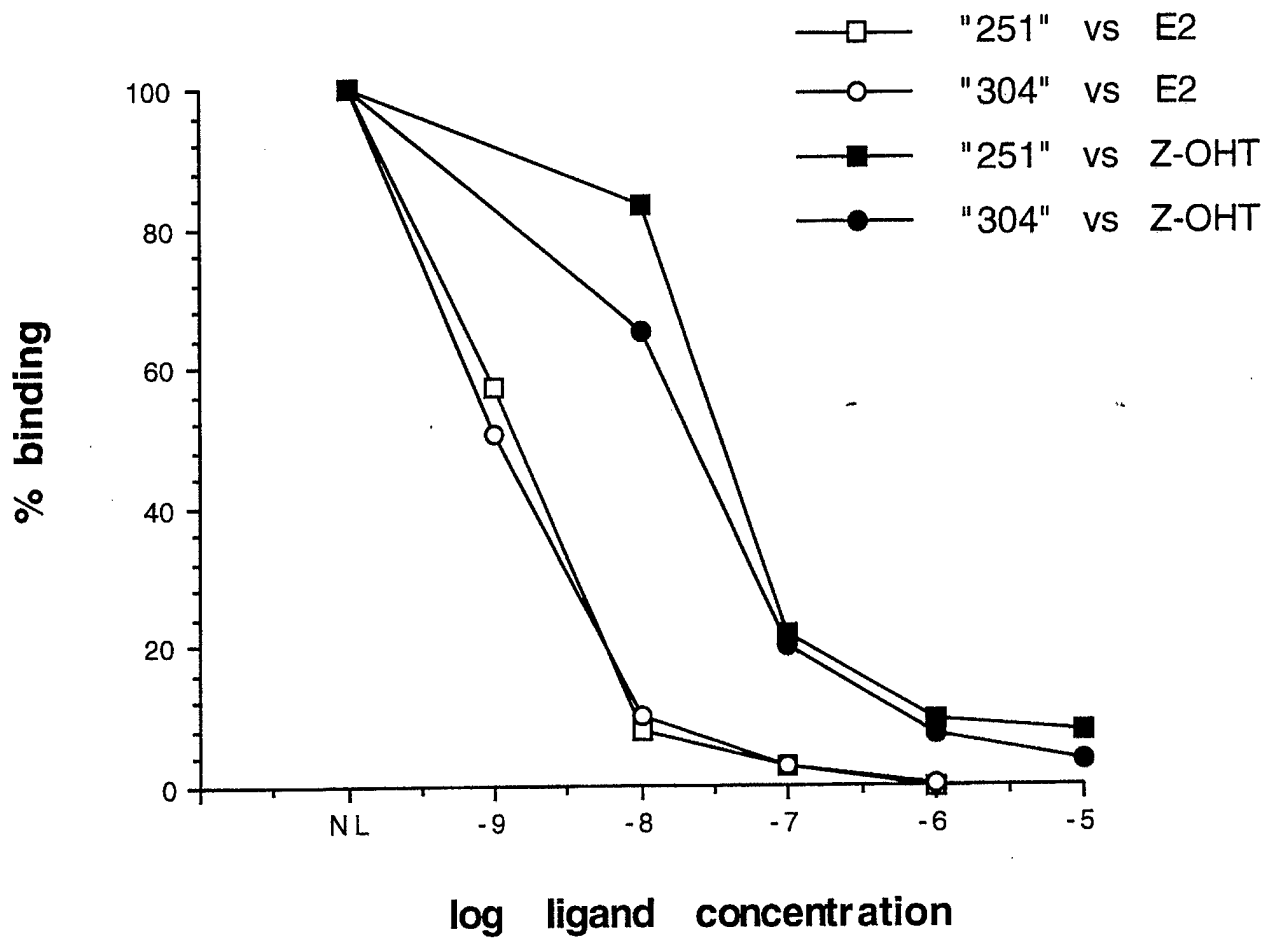
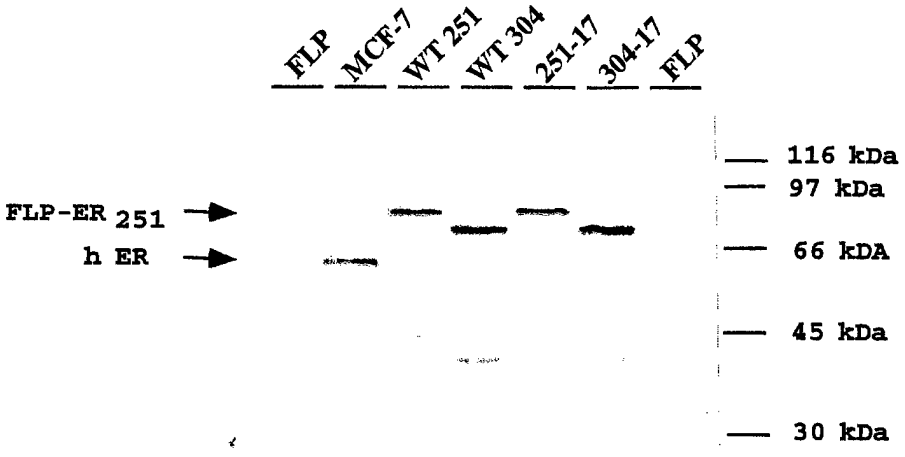
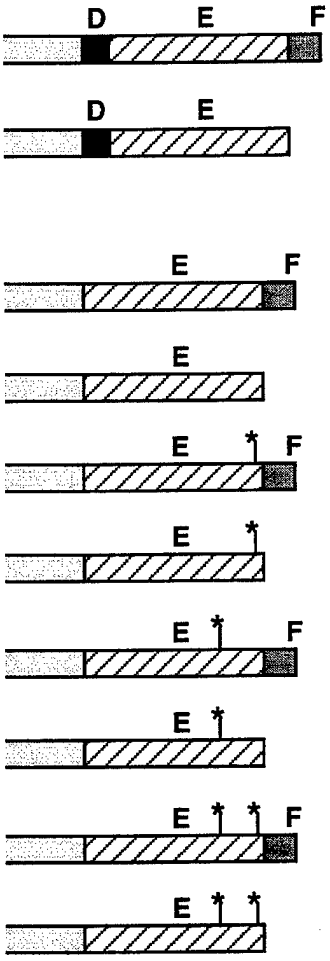

WT 251
WT 304

Figure 3.



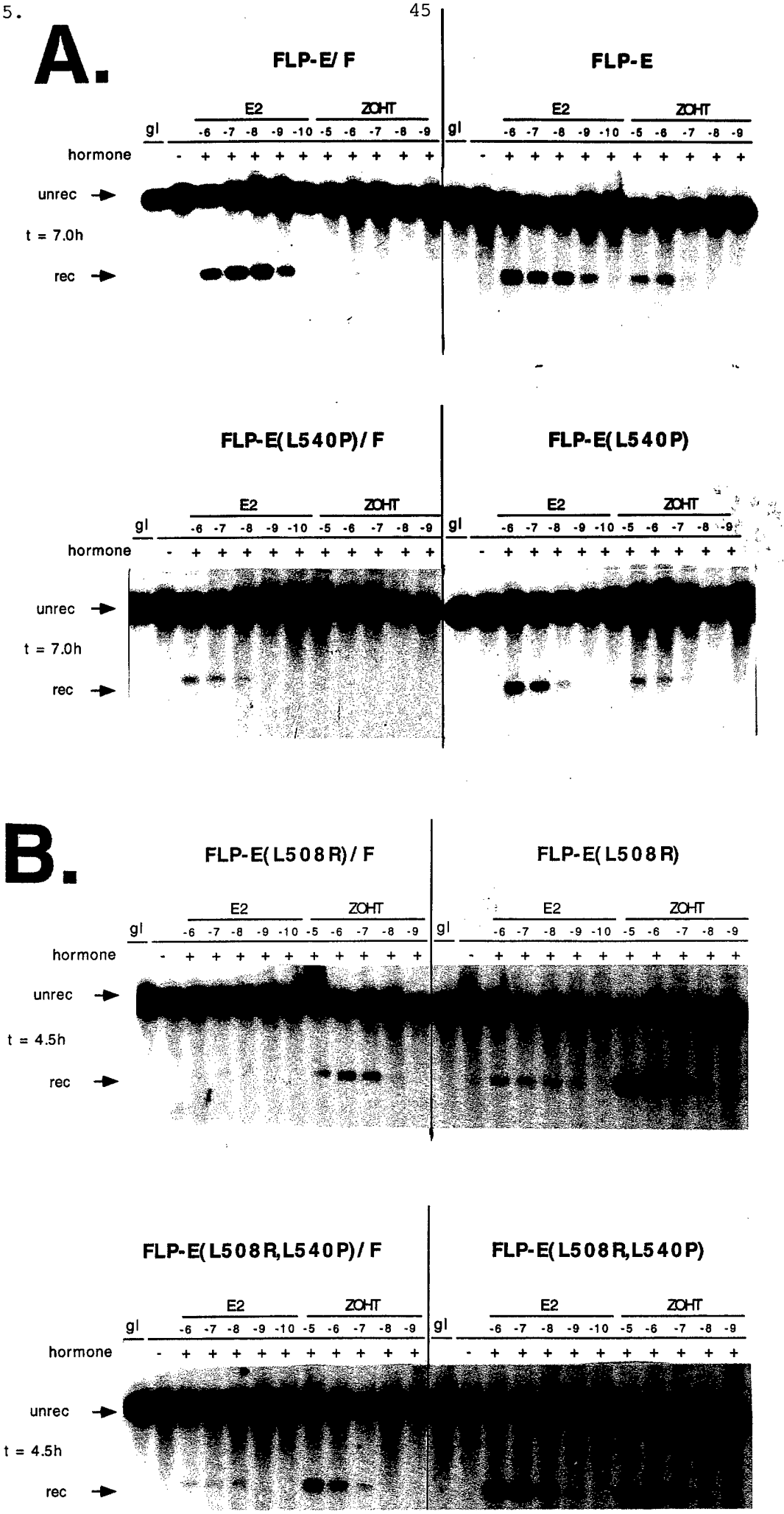
α ER (aa 576-595)



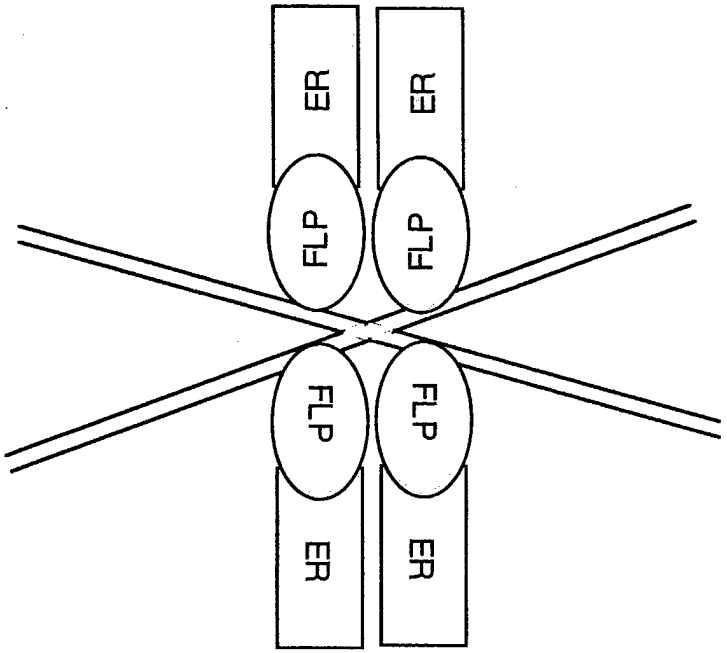


	IC_{50} (nM)			
	E2	ZOHT	RAL	ICI
FLP-D/ E/ F	0.6	1.6	1.5	1.5
FLP-D/ E	0.8	2.1	1.8	1.9
FLP-E/ F	0.8	2.0	1.5	2.3
FLP-E	0.5	1.3	1.2	1.7
FLP-E(L540P)/ F	0.8	0.6	0.7	1.0
FLP-E(L540P)	0.9	0.6	1.0	0.7
FLP-E(L508R)/ F	0.6	1.4	2.5	2.0
FLP-E(L508R)	0.7	0.8	2.2	1.3
FLP-E(L508R,L540P)/ F	0.8	0.5	0.8	0.6
FLP-E(L508R,L540P)	0.6	0.5	0.7	0.6

Figure 5.


A.
FLP-E/ F
FLP-E
E2
ZOHT
gl
-6 -7 -8 -9 -10 -5 -6 -7 -8 -9
hormone
- + + + + + + + + + +
unrec
t = 7.0h
rec
FLP-E(L540P)/ F
FLP-E(L540P)
B.
FLP-E(L508R)/ F
FLP-E(L508R)
t = 4.5h
FLP-E(L508R,L540P)/ F
FLP-E(L508R,L540P)

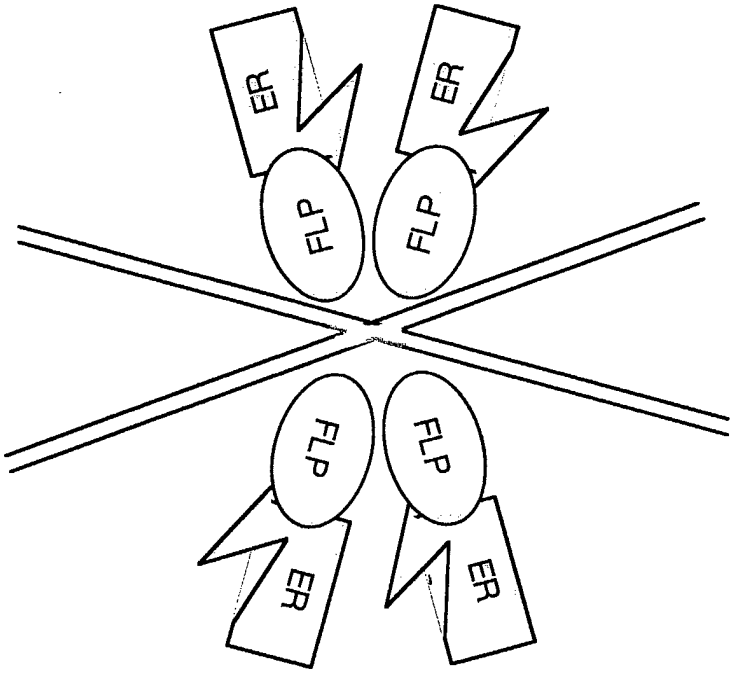
FLP Recombinase - Estrogen Receptor Fusion Protein Requires Hormones for Activity



ACTIVE

INTERMEDIATE

WITH HORMONES



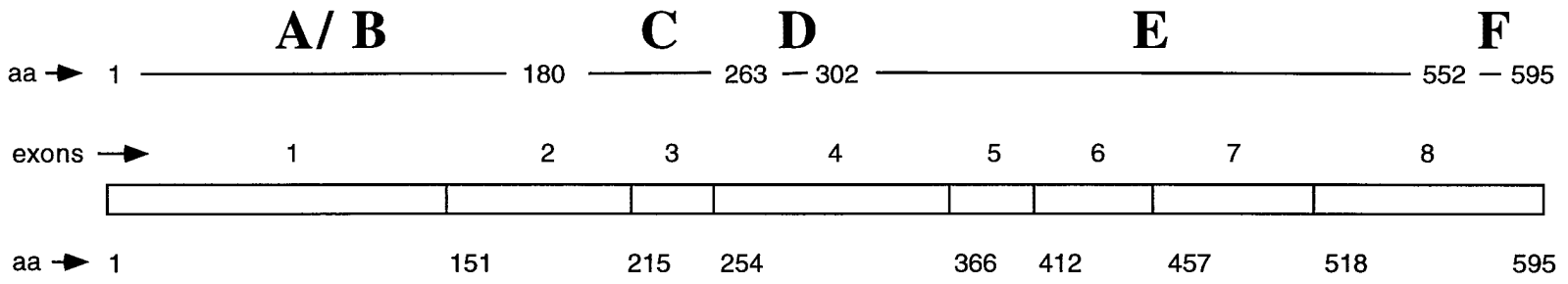
INACTIVE

WITH ANTIHORMONES

Figure 6.

A.

Human Estrogen Receptor α



FLP Recombinase Activity Depends on Properly Folded ER Binding Domain, Induced by Hormones

B.

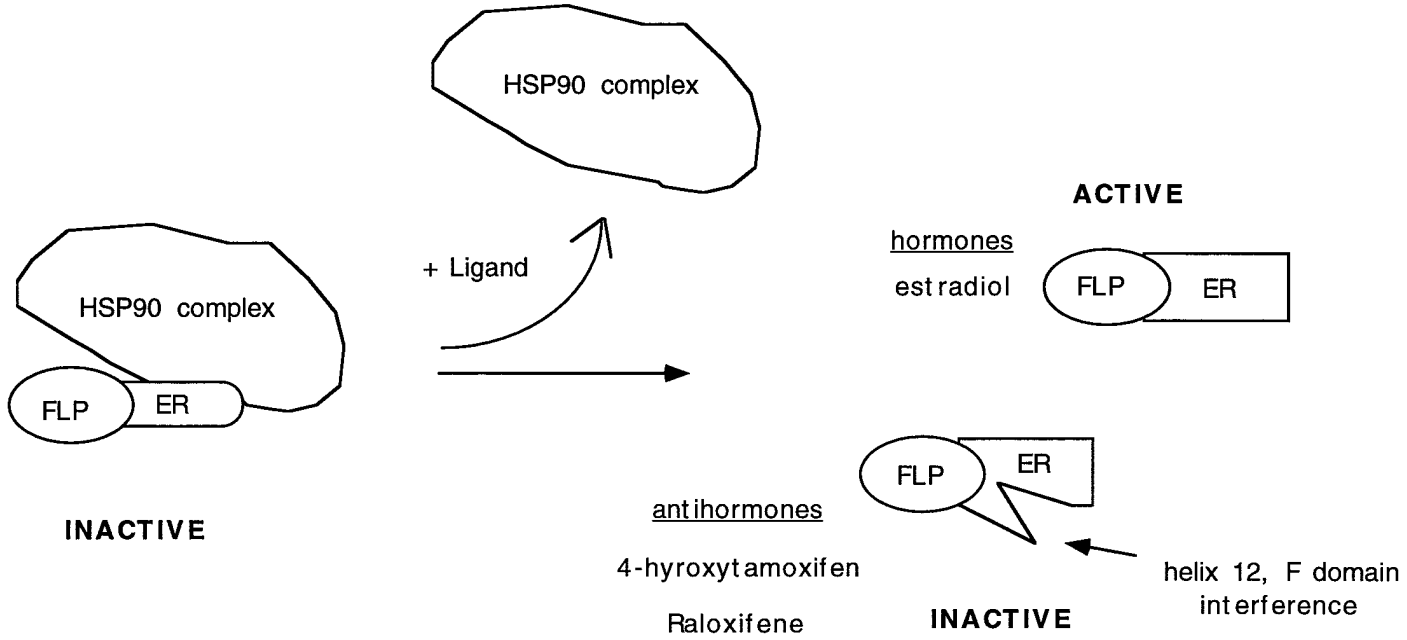
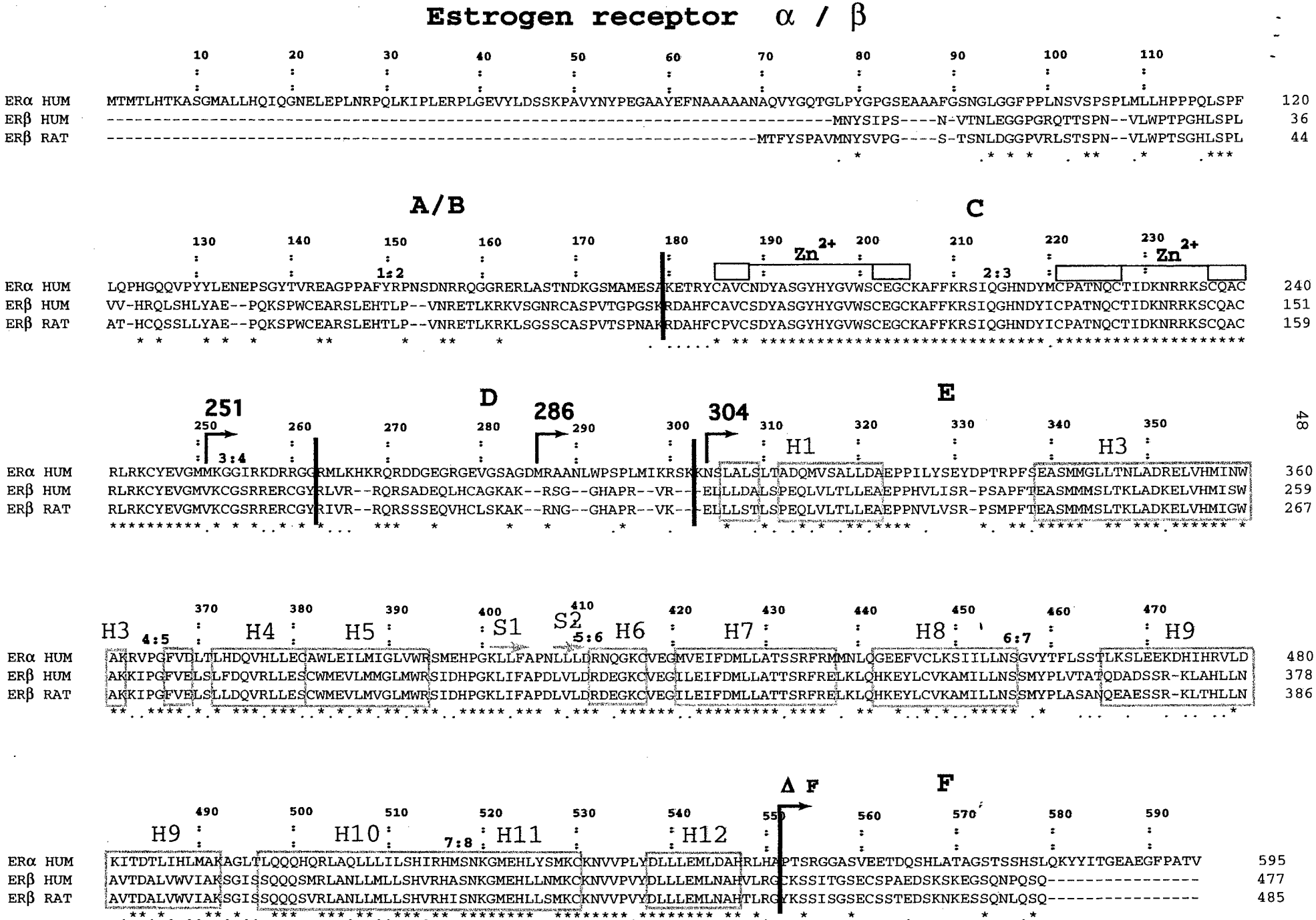


Figure 8.


Estrogen receptor α / β
A/B
C
D
E
F
Zn2+
Zn2+
H1
H3
H3
H4
H5
S1
S2
H6
H7
H8
H9
H9
H10
H11
H12
ΔF
ERα HUM
ERβ HUM
ERβ RAT

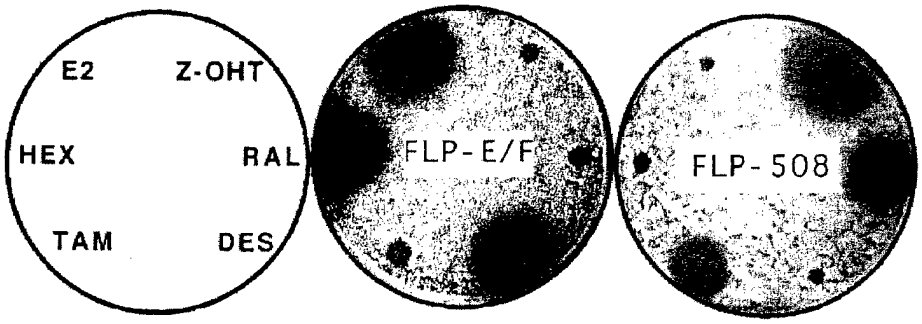
A.

Step 1. Select against recombination in the presence of E2.

Step 2. Screen for recombination in the presence of Z-OHT.

Step 3. Isolate the FLP-ER plasmid and retransform for plate assay and ligand activity.

B.



C.

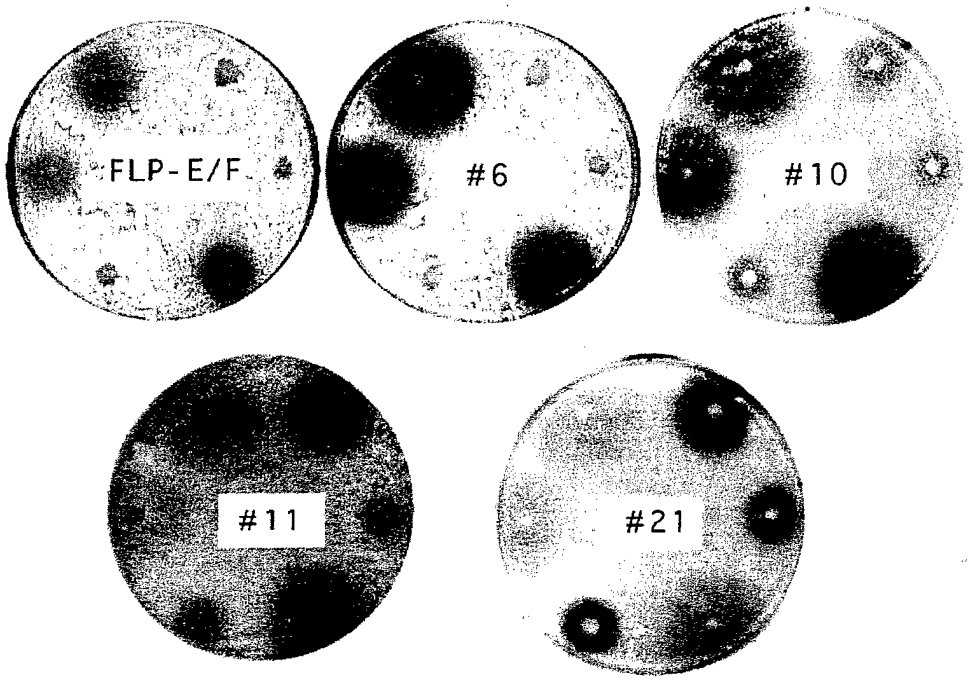
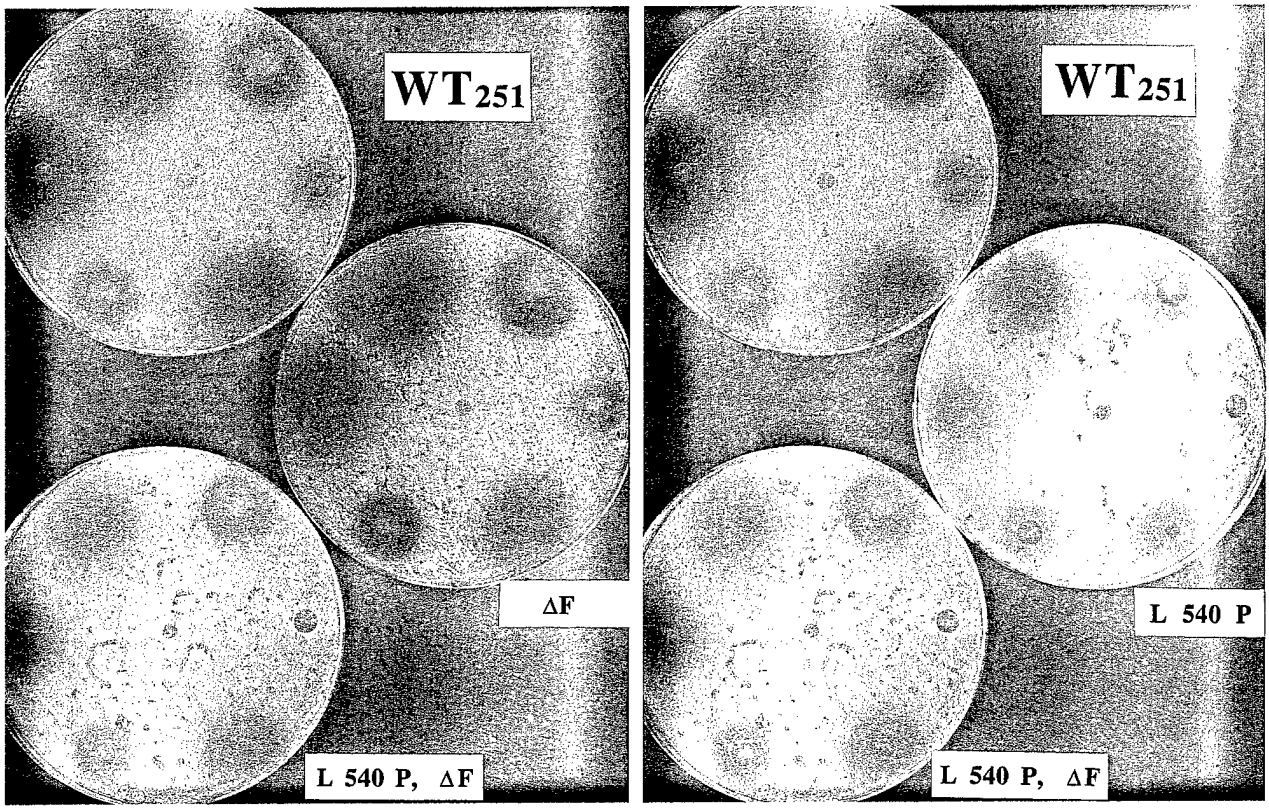


Figure 10.



Steroid Receptor Activity Depends on Properly Folded Ligand Binding Domain, Induced by Hormones

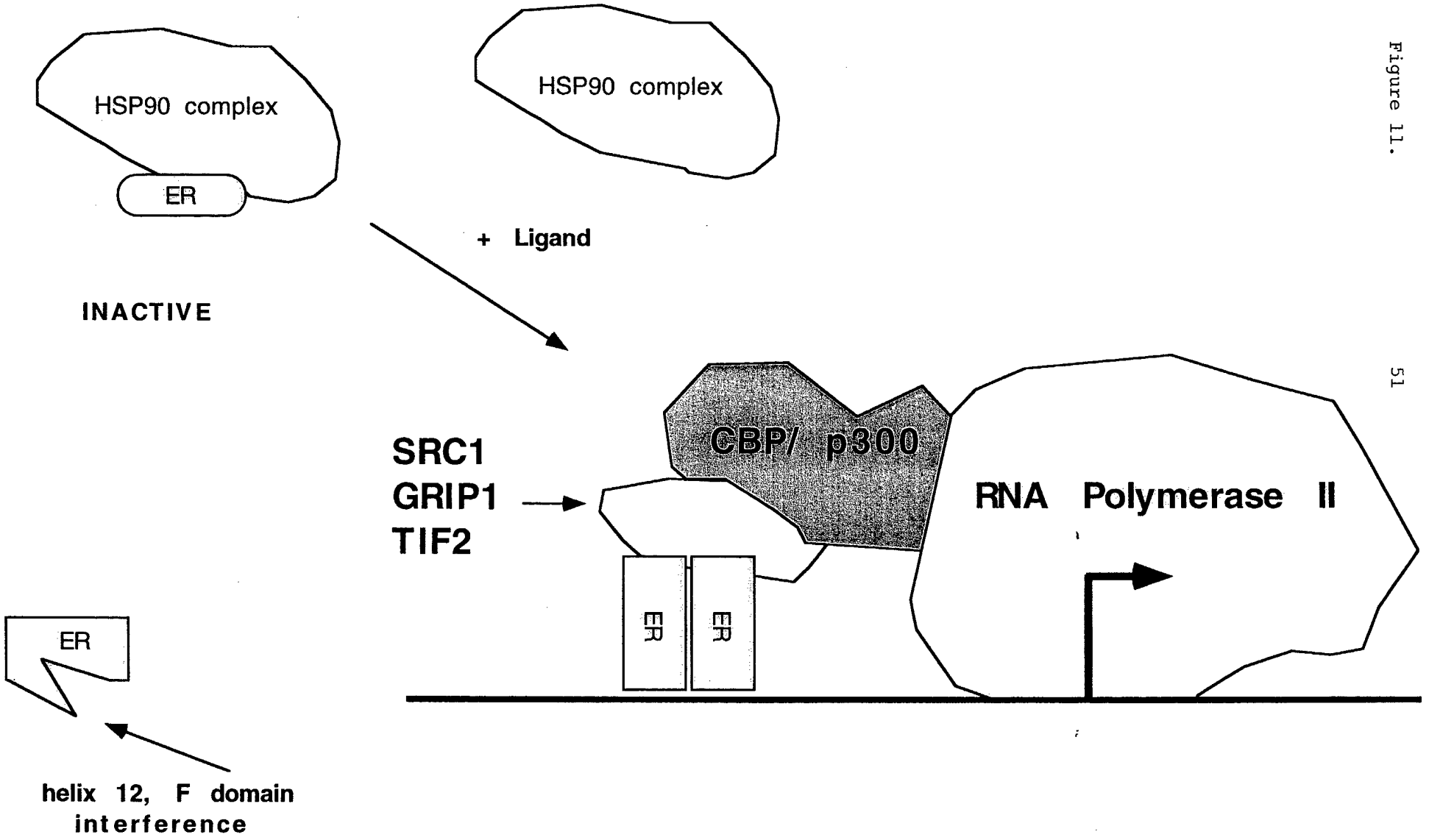


Figure 11.

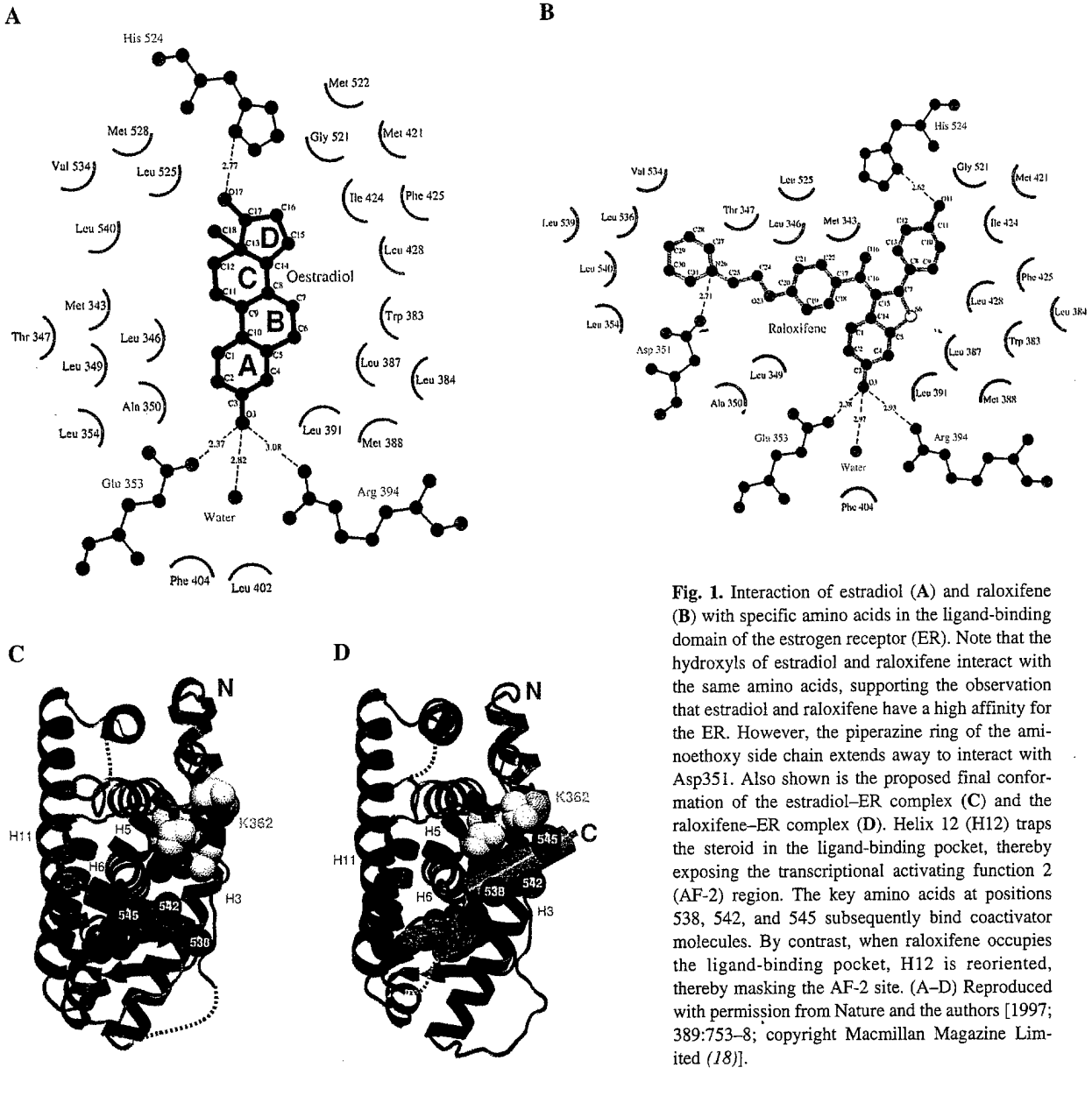


Fig. 1. Interaction of estradiol (**A**) and raloxifene (**B**) with specific amino acids in the ligand-binding domain of the estrogen receptor (ER). Note that the hydroxyls of estradiol and raloxifene interact with the same amino acids, supporting the observation that estradiol and raloxifene have a high affinity for the ER. However, the piperazine ring of the aminoethoxy side chain extends away to interact with Asp351. Also shown is the proposed final conformation of the estradiol–ER complex (**C**) and the raloxifene–ER complex (**D**). Helix 12 (H12) traps the steroid in the ligand-binding pocket, thereby exposing the transcriptional activating function 2 (AF-2) region. The key amino acids at positions 538, 542, and 545 subsequently bind coactivator molecules. By contrast, when raloxifene occupies the ligand-binding pocket, H12 is reoriented, thereby masking the AF-2 site. (A–D) Reproduced with permission from Nature and the authors [1997; 389:753–8; copyright Macmillan Magazine Limited *(18)*].

The following papers have resulted from the work..

1. Stewart, A.F., Logie, C. and Nichols, M. (1996) Regulation of nuclear receptors by agonists and antagonists. *Curr Opin Endocrin & Diabetes* **3**: 397-402.

2. Nichols, M., Rientjes, J.M.J., Logie, C. and Stewart, A.F. (1997) FLP recombinase/ estrogen receptor fusion proteins require the receptor D domain to convey responsiveness to antagonists, but not agonists. *Molec. Endocrinol.* **11**: 950-961.

3. Nichols, M., Rientjes, J.M.J. and Stewart, A.F. (1998) Different positioning of the ligand binding domain helix 12 and the F domain of the estrogen receptor accounts for functional differences between agonists and antagonists. *EMBO J.*, **17**, 765-773.

4. Logie, C., Nichols, M., Myles, C., Funder, J.W. and Stewart, A.F. (1998) Positive and negative discrimination of estrogen receptor agonists and antagonists using site-specific DNA recombinase fusion proteins. *Molec. Endocrinol.* **12**, 1120-1132.

Salaries paid by this grant to Mark D. Nichols

Conferences and Abstracts

March, 1998, Keystone Symposium; Lake Tahoe, California
Steroid and Nuclear Receptor Gene Family
"Different positioning of the LBD helix 12 and F domain of the estrogen receptor accounts for functional differences between agonists and antagonists."

October 31- November 4, 1997; Washington, D.C.
U.S. Army Medical Research and Materiel Command (USAMRMC)
Breast Cancer Research Program: An Era of Hope
"A Genetic Screen for Ligand Binding by the Human Estrogen Receptor"

October 4-7, 1997; EMBL, Heidelberg, Germany;
Chromatin and Epigenetic Regulation

July 28-29,1997, Philadelphia, Pennsylvania
Nuclear Hormone Receptors: Targets for Therapeutic Intervention
"Functional properties of estrogen receptor ligand binding domains assayed by FLP recombinase fusion proteins"

August, 1996
EMBL Meeting on Transcription; Heidelberg, Germany
"Functional properties of steroid receptor ligand binding domains assayed by FLP recombinase fusion proteins"

March, 1996, Keystone Symposium; Lake Tahoe, California
Steroid/ Thyroid/ Retinoic Acid Gene Family
"Using FLP recombinase/ligand binding domain (FLP/LBD) fusion proteins in *S. cerevisiae* to assay hormone interactions and functions"